A study of gene expression during ripening in Capsicum annuum fruit

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To my mother.

Contents.

Abstract.

Fruit ripening has been shown to be a developmental process in tomato and avocado, involving alterations in gene expression. Fruit ripening in *Capsicum annuum* was investigated. During ripening there is a chloroplast to chromoplast transition, which is a period of great structural and metabolic change. Chromoplasts have been shown to be inactive in protein synthesis, and the transition is therefore believed to be brought about by nuclear gene expression and transport of proteins to the plastids. The pigmentation and ultrastructural characteristics of *Capsicum annuum* were studied during ripening and revealed dramatic changes. The protein complements of normal and mutant varieties were studied by sodium dodecyl sulphate polyacrylamide gel electrophoresis, isoelectric focusing, and two dimensional gel electrophoresis. A group of ripening associated proteins was identified using each method. A protocol was developed for the extraction of RNA from fruit, and the RNA population studied by *in vitro* translation, again revealing a group of ripening associated proteins. Some of this group were tentatively identified as the larger precursors of 'ripening' chromoplast proteins. The techniques for isolation of a ripening associated cDNA clone were established. The results corroborate previous findings that ripening is a developmental process, involving changes in gene expression.

1.1.00 General Introduction.

1.1.01 Introduction.

The aim of this work was to investigate the changes occurring in pepper fruit during ripening, particularly the molecular biology, and eventually to use the ripening pepper fruit as a model to study differential gene expression in plants. The sweet bell pepper, *Capsicum annuum* var. Bellboy was chosen as a system, for the following reasons: (i) it is a convenient glasshouse crop and can be produced all the year round; (ii) sweet peppers are commercially important, especially in Holland and the USA; (iii) some interesting ripening mutants exist and (iv) the sweet pepper is a non-climacteric fruit which is unusual for this kind of investigation (Saltveit, 1977). (The climacteric is the name for the respiratory surge which accompanies ripening in some fruit, and it will be discussed later.)

C.annuum belongs to the Order Scrophulariales, which is a large group containing 18 families, 870 genera and over 11 800 species. Peppers are part of the potato family, the Solanaceae, which also contains the tomato and the aubergine. They were introduced to Europe by Columbus in 1493 (Heister, 1969) from the New World where they had been used for centuries. The earliest evidence that peppers were part of the diet was obtained from a study of coprolites in South America,

which revealed that peppers were being eaten by humans as long ago as 7000 BC. Evidence of cultivation was found at Huaca Pieta in South America dated at about 2500 BC; the cultivated fruits were larger than the wild type, indicating selective breeding. Peppers of various sorts now occupy large areas of fertile ground; 1041 000 HA in 1985 (FAO yearbook, 1985).

Ripening of a fruit is a process designed to turn it into an active seed container. Fruits which are destined to be eaten by animals become more edible. This often involves softening, the production of aromatic smells and flavours, and recognizable ripe colour. The colouration is usually brought about by the deposition of carotenoids in the fruit cells. Reviews of the ripening process have been written including those of: Grierson *et al.* (1981); Grierson and Covey (1984); Grierson (1986).

Early ideas about ripening centred around the supposition that ripening was a degradative process, involving a breakdown of cellular organization and the release of hydrolytic enzymes. Later an alternative hypothesis was developed which proposed that ripening was an active developmental process, involving specific gene expression (Grierson *et al.*, 1985).

1.1.02 Ripening patterns.

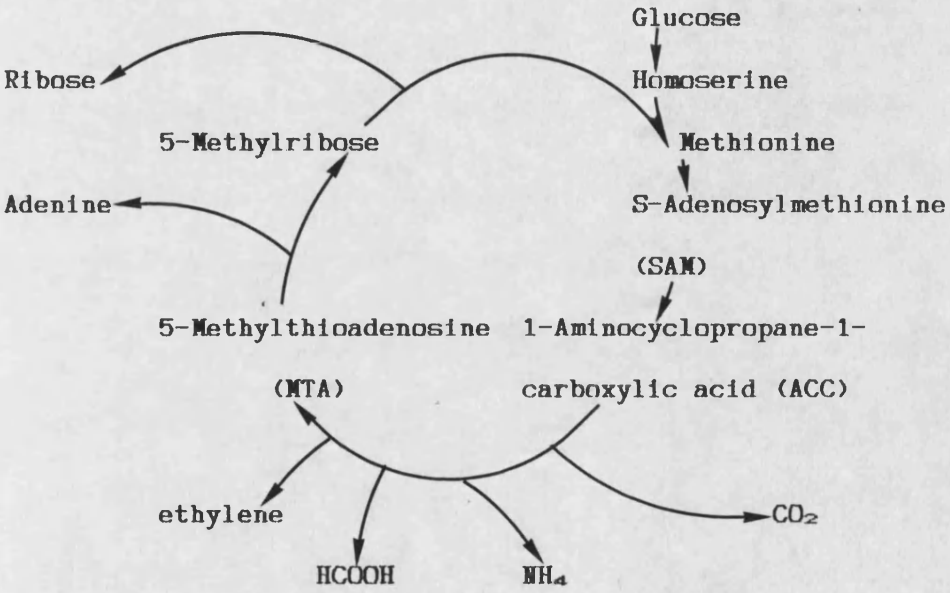
In the literature, ripening fruits are divided into two types according to their pattern of ripening. Such a division is not absolute, and differing degrees of each type of pattern are seen. The two types are described as climacteric and non-climacteric, the difference between them being that climacteric fruits show a rise in their rate of respiration prior to ripening, and autocatalytic ethylene production, whereas non-climacteric fruits do not. Climacteric fruits include the avocado (Christoffersen *et al.*, 1982), the tomato (Grierson and Covey, 1984) and the hot pepper (Gross *et al.*, 1986). Climacteric fruits respond to ethylene with positive feedback on ethylene biosynthesis i.e. the presence of ethylene induces the cells to produce an ethylene surge (Biale and Young, 1981). Non-climacteric fruits do not have this surge; this is believed to be due to a lack of the ethylene inter-mediate 1-aminocyclopropane-1-carboxylic acid (ACC). It is thought that cultivation led to the selection of non-deciduous fruit which were also non-climacteric. The Mexican Bird Pepper, believed to be the progenitor of our sweet pepper, is both deciduous and climacteric (Heister, 1969).

1.1.03 Ripening and ethylene.

Ethylene is a gaseous plant hormone produced in the plant from its precursor methionine. The biosynthesis of ethylene is summarized in Figure 1. Ethylene seems to stimulate and co-ordinate the ripening process in climacteric fruit (Grierson

and Covey, 1984). Endogenous ethylene reaches a threshold level which is then sufficient to stimulate a surge of ethylene production from the fruit cells, by a positive feedback mechanism. The surge of ethylene is dependent upon a preceding surge of the ethylene intermediate, ACC.

Figure 1. The biosynthesis of ethylene.



(from Yang, 1981)

Ethylene has been shown to have various effects on fruit cells. In tomatoes it was shown that ethylene stimulates the synthesis of lycopene, a carotenoid, and also the production of polygalacturonase (PG), a wall-softening enzyme (Grierson and Tucker, 1983). Ethylene also induced changes in the number and size of polyribosomes and altered the mRNA population in ripening tomatoes (Tucker and Laties, 1984). The same effect was also noticed in carrots by Christoffersen and Laties,

(1982). Although ethylene initiates ripening in climacteric fruit it does not necessarily initiate the respiratory climacteric which sometimes precedes the ethylene surge (Biale and Young, 1981). Very little is known of the mechanism of ethylene action. Its effect of limiting the production of normal proteins in favour of ripening specific ones may be significant in ripening initiation as may be its effect of reducing membrane turnover (Baker *et al.*, 1985). Ethylene has been shown to be a 'perturbor' of membranes, allowing greater permeability for example. This may contribute, in that precursors for ripening associated metabolism (such as carotogenesis) may enter specific compartments (such as the chromoplast) more easily (Schneider *et al.*, 1977). Changes may also be brought about by altering compartmentalization, vacuolation or electrical impedance.

Ethylene is thought to bind to a metalloprotein initially (Christoffersen and Laties, 1982) and is found to require the presence of oxygen in order to act (Rhodes, 1980). The transient nature of ethylene retention indicates that binding is probably by weak van der Waals forces. Lincoln *et al.* (1987) studied the responses of tomato ripening specific mRNAs to ethylene, and showed that some gene expression increased at low basal levels of ethylene and other ripening specific gene expression increased at higher levels of ethylene. The authors concluded that along with variation in ethylene levels, there were variations in ethylene sensitivity for individual genes.

Deikman and Fischer (1988) identified an ethylene responsive gene in tomato, and showed that the responsiveness correlated with the interaction of a DNA-binding protein with sequences both 5' and 3' to the coding region of the gene. A possible mode of regulation has therefore been elucidated.

Silver ions act as inhibitors of ethylene action in climacteric tomato fruits (Tucker and Brady, 1987), and included among the effects of dosing with silver thiosulphate were prevention of the following: chlorophyll degradation; PG accumulation and activity; lycopene accumulation; and invertase accumulation and activity. The silver ions are thought to interfere with ethylene action by binding or interfering at specific sites, for example on the metalloprotein that the ethylene is believed to bind to.

Non-climacteric fruit seem unable to produce the ACC surge and consequently the ethylene surge, although very little is known about the mechanisms involved. Saftner and Baker (1987) studied the uptake of ACC and its structural analogue α-aminoisobutyric acid (αAIB). They found that the bulk of αAIB is sequestered into the vacuole, which is believed to be the site of ethylene synthesis, and suggested that the rate of ethylene production may be related to the ability of the cell to transport and sequester ACC. Non-climacteric fruits may possess a deficient ACC transport system which prevents the accumulation of ACC, and hence prevents an ethylene surge. An

increase in ABA (abscisic acid) occurs during ripening and it is possible that ABA is the ripening promoter in non-climacteric fruit (Solomos and Laties, 1973). In the non-climacteric strawberry fruit, inhibitors of ethylene action and synthesis have no effect on the rate of ripening (Given *et al.*, 1988), rather, the decline in auxin production from the maturing achenes seems to induce the process.

1.1.04 Ultrastructural changes during ripening.

Ripening fruit cells undergo many changes at the ultrastructural level. Overall cell size has been shown to increase (Rhodes, 1980) reaching 150-700 μm in diameter in some fruit; such cells are among the biggest plant cells known. Most of the changes are found to take place in the plastids. Kirk and Juniper (1967) and Simpson *et al.* (1976) studied the ultrastructure of *C.annuum* plastids during ripening. During this period the chloroplasts of the green fruit are converted into the chromoplasts of the red fruit. This involves breakdown of the typical granal structure of the thylakoids and lamellar junctions into a 'thylakoid plexus' containing fibrils and plastoglobuli. There is a reduction in the size and number of the starch grains, and development of lipid globules. The ability to photosynthesize is lost and the chromoplast is turned over to the synthesis of carotenoids, believed to constitute the globules and found in crystalline form between the thylakoids. In the tomato, wall-softening

occurs as the enzyme polygalacturonase solubilizes the middle lamella (Crookes and Grierson, 1983), although the extent of this softening has been questioned by Seymour *et al.* (1987). Ripening mutants which show reduced or altered carotenoid content also show reduced or altered ultrastructural changes. One of the purposes of the changes in ultrastructure seems to be to provide an environment suitable for the production of carotenoids.

1.1.05 Metabolic changes during ripening.

In correlation with the increase in size of the fruit cells the walls undergo changes in composition. A survey of several fruits by Gross and Sams (1984) showed that in general there was a net loss of non-cellulosic neutral sugar residues, mainly galactose and arabinose. In hot pepper the loss of galactose and arabinose correlated with a 50-fold increase in β-galactosidase activity (Gross *et al.*, 1986). Unlike tomato there was no detectable level of polygalacturonase activity. The role of this enzyme has recently been questioned by Seymour *et al.* (1987), who showed that the degradation of polyuronides in the cell wall is much less extensive than originally believed and shows no close correlation with polygalacturonase or pectinase levels in normal and mutant tomatoes.

Most of the studies performed have involved climacteric fruit, in particular the tomato and avocado, concentrating on the respiratory surge itself and the response of climacteric fruits to ethylene. Such investigations are pertinent to a study of a non-climacteric fruit because they lead to an increased understanding of the ripening process, and because even non-climacteric fruit may exhibit the same characteristics although to a lesser extent. The presence of climacteric behaviour in tomato and hot pepper (a different variety of *C.annuum*) and the absence in sweet pepper, suggests a fairly simple difference between climacteric and non-climacteric fruit.

Solomos and Laties (1974 and 1976) studied the respiration of ripe avocado and found that the respiratory climacteric, anoxia and cyanide (HCN) had the same effect on respiration, that is, these factors increased the rate of glycolysis and ATP levels. In tissues other than ripening fruit, HCN produces a fermentation response, a fact which led the authors to suggest that ripe fruit must possess an HCN-resistant electron transport path-way. HCN also acts to stimulate ethylene biosynthesis, and it is thought that ethylene perturbs the membranes allowing greater permeability to glycolytic precursors. The rate of glycolysis was found to be proportional to the rate of phosphate leakage from the vacuole via the permeablized tonoplast. Bennett *et al.* (1987) suggest that the respiratory climacteric in avocado fruit may be

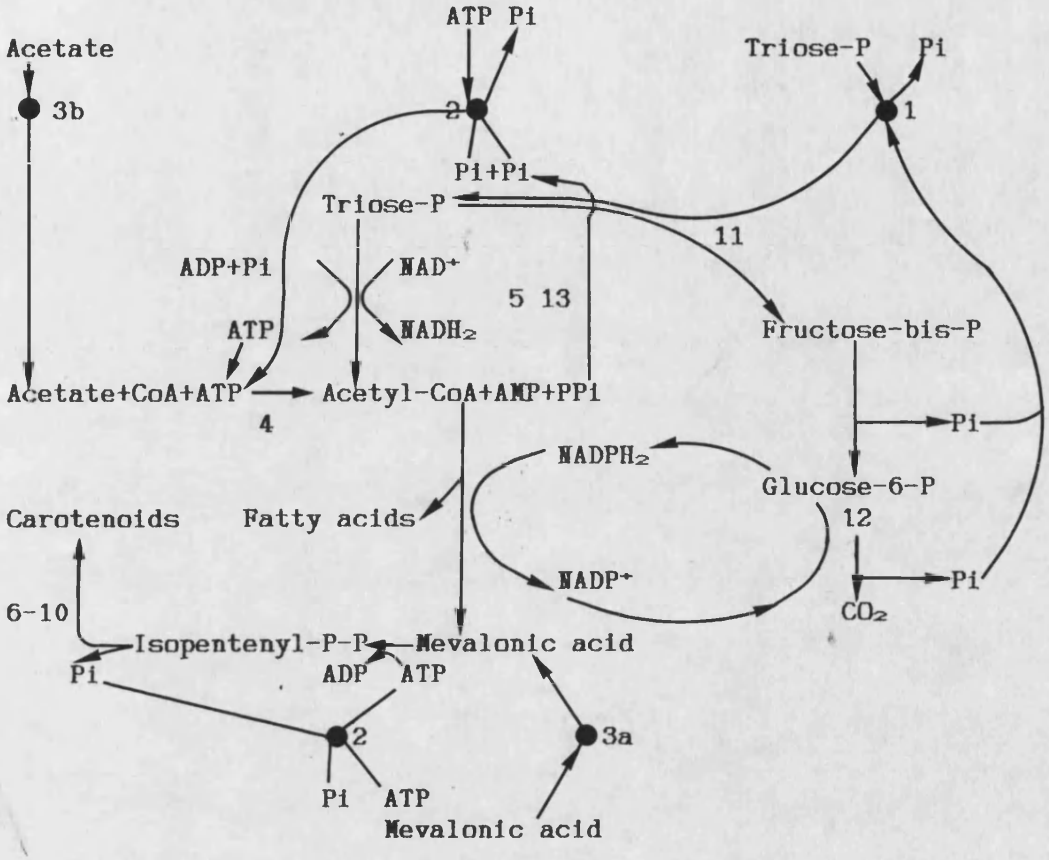
caused by increases in activity of phosphofructokinase or pyrophosphate : fructose-6-phosphate phosphotransferase, the latter possibly being stimulated by a 90% increase in the levels of fructose-2,6-biphosphate. Another glycolytic enzyme, fructose-1,6-biphosphate aldolase was found to increase in ripe tomatoes at the mRNA level (Piechulla, 1988), during a study of the levels of several nuclear and plasid encoded mRNAs during ripening. Only the presence of an HCN-resistant electron pathway seems to explain the high levels of ATP, and it was suggested by Solomos and Laties (1974 and 1976) that implementation of this pathway de-controls oxidative phosphorylation. Why such a pathway should be implemented is unknown. The respiratory climacteric can be viewed as a process which continues alongside ripening rather than being implicated as a cause; this helps to explain the lack of correlation between the appearance of the climacteric and the rest of the ripening changes, exemplified by ripening in non-climacteric fruit.

Biale and Young (1981) divide the metabolic aspects of ripening into degradative and synthetic categories. Included in the degradative category are the breakdown of chlorophyll, the loss of photosynthesis, the hydrolysis of starch grains, the initiation of some membrane leakage, the solubilization of pectins and the release of hydrolytic enzymes. In the synthetic category are included the maintenence of mitochondria, the preservation of selected membranes, the

enhancement of ripening specific transcription and translation, the initiation of the ethylene synthesis pathway and the production of carotenoids and flavours. In spite of the differentiation of chloroplasts into chromoplasts it is believed that the plastid machinery of protein import remains functional. Non-photosynthetic plastids were shown to incorporate correctly and process a protein normally confined to photosynthetic plastids (de Boer *et al.*, 1988) indicating that processing and transport mechanisms were acting similarly regardless of the differential state.

Ziegler *et al.* (1983) investigated the changes taking place in plastids of *C.annuum* fruit pericarp cells as ripening took place. They found that chlorophyll was reduced to zero, and therefore photosynthesis likewise reduced, with the enzymes of the Calvin cycle decreased in activity by 80%. Glycolysis was found to be increased however. The chromoplast was turned over to the production of fatty acids and carotenoids. It is not known how the breakdown of the photosynthetic apparatus is regulated although ethylene is shown to accelerate the breakdown of chlorophyll into small colourless fragments (Goldschmidt, 1980), neither is it known how much the chlorophyll breakdown products contribute to the chromoplast's new functions (e.g. pigment production). In contrast to the chloroplast the chromoplast is a metabolically heterotrophic organelle, depending on the cytosol for organic precursors and energy. The metabolism of a chromoplast is shown in Figure 2.

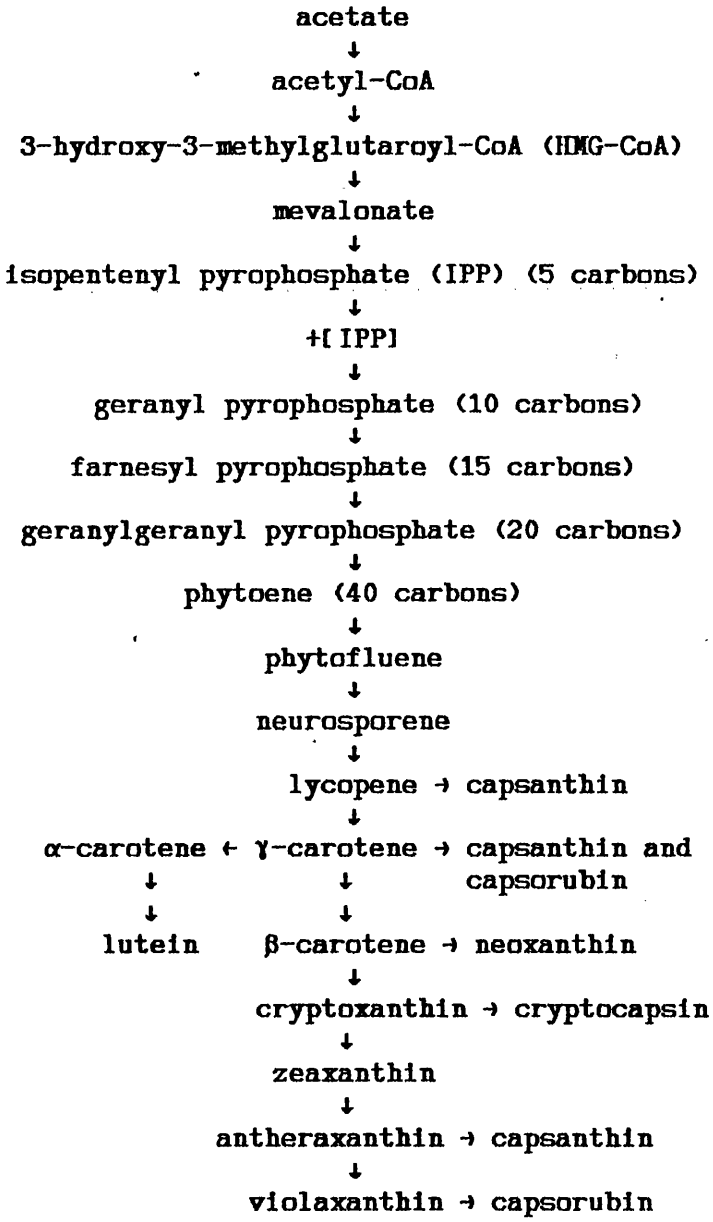
Figure 2. The metabolism of a chromoplast.



(from Ziegler *et al.*, 1983)

Figure 2. 1, phosphate translocator; 2, adenylate translocator; 3, acetate-mevalonate permeation into the plastid in the transition phase (Schneidner *et al.*, 1977); 4, acetyl-CoA synthetase; 5, glycolysis; 6 to 10, carotenoid biosynthesis from isopentenyl pyrophosphate (Beyer *et al.*, 1980; Kreuz *et al.*, 1982); 11, aldolase (Ziegler *et al.*, 1983); 12, glucose-6-phosphate dehydrogenase (Ziegler *et al.*, 1983); 13, NAD^+-dependent triosephosphate dehydrogenase (Ziegler *et al.*, 1983).

Figure 3. The terpenoid pathway.



(from Manitto, 1981; Davies *et al.*, 1970)

1.1.06 Carotogenesis.

Carotenoids are unsaturated polyhydrocarbons with aliphatic or alicyclic hydrocarbon chains, and their oxygenated derivatives (Britton, 1985). The chromophore is formed from a series of conjugated double bonds. They are produced in plants by the terpenoid pathway, and cannot be made by animals, any carotenoids found in animal cells reached those cells via the diet. The production of carotenoids by the terpenoid pathway is summarized in Figure 3. In green tissues there are a number of carotenes, the main ones being β-carotene, lutein, violaxanthin and neoxanthin. During ripening there is massive synthesis of the keto-xanthophylls (keto group on C1) mainly capsorubin, capsanthin and cryptocapsin. Candela *et al.* (1984) performed thin layer chromatography (TLC) on the extracted pigments of *C.annuum* throughout ripening and showed the alteration of carotenoid content as the fruit passed from unripe to ripe. The chromoplast becomes permeable to the precursors of carotenoid biosynthesis, such as mevalonate and acetate which are translocated from the cytosol into the plastid (Schneidner *et al.*, 1977).

The carotenoids of ripening mutants are found to be altered or deficient compared with the wild type (Davies *et al.*, 1970) in conjunction with abnormal ultrastructural features.

The pepper chromoplast contains all the enzymes necessary for carotenoid biosynthesis. Most of the work on the elucidation of enzyme sites within the plastid has been done by Camara and co-workers. By separating *C.annuum* chromoplast stromal and membrane fractions and analysing enzyme activity in each, it has been demonstrated that phytoene synthase is a stromal (or peripheral) enzyme (Camara, 1984), and all the following reactions of desaturation and cyclization take place on the plastid membranes (Camara *et al.*, 1982, 1985; Camara and Dogbo, 1986).

Chromoplasts are also present in the petals of flowers. In daffodil, the chromoplast fractions were analysed by Kreuz *et al.* (1982), and it was found that intermediates of carotenoid biosynthesis were only produced if the stromal and thylakoidal fractions were combined. Phenyl transferase and phytoene synthase were found to be peripheral membrane proteins and the isomerase, cyclase and dehydrogenase found to be integral membrane proteins. In conjunction they form an assembly line of tight spatial integrity.

The production of carotenoids renders the chromoplast a metabolically heterotrophic organelle, and it is therefore interesting to speculate on the purpose of these pigments. In other plant tissues carotenoids act as accessory pigments in photosynthesis, as scavengers of unwanted oxygen, as antagonists of potentially lethal photosensitization of

chlorophyll; as photoreceptors; and to protect delicate tissues from radiation. In fruit their most likely function is that of advertizing to animals and birds that the fruit is ripe, and thereby promoting dispersal of the seeds. Plant carotenoids provide a valuble source of pro-vitamins and pigments for animals and this makes the fruit very attractive, to these potential agents of seed dispersal.

1.1.07 Control of ripening.

Ripening involves many structural and metabolic changes, the most obvious taking place in the plastid; carotogenesis for example is contained entirely within the plastid. It is important to try and understand the contributions of the two genetic systems, nuclear and chloroplast, to the process of ripening and in pursuit of this four main lines of work have been looked at; antibiotic studies; other studies of plastid protein synthesis during ripening; mutation studies; and comparisons of total and polyadenylated RNA.

Benedict *et al.* (1985) treated *Citrus* with the substituted tertiary amine, 2-(4-methylphenoxy)triethylamine (MPTA) and found that it stimulated the synthesis of phytofluene and lycopene. The synthesis was shown to be inhibited by the mushroom toxin, α-amanitin, which inhibits nuclear DNA-dependent RNA polymerase; by cordycepin, which inhibits polyadenylation of the primary transcript; and by anisomycin

and cycloheximide, which prevent protein synthesis on cytoplasmic 80S ribosomes. Therefore the synthesis of these carotenoids seems to require nuclear transcription, polyadenylation of the primary transcript and translation on 80S ribosomes. Surzycki (1973) found that treatment of *Chlamydomonas reinhardtii* with rifampicin inhibited prokaryotic DNA-dependent RNA polymerase (i.e. the type of RNA polymerase active in plastid transcription), and the loss of ribosomal protein meant the organism could no longer photosynthesize. Sirevåg and Levine (1973) treated *Chlamydomonas* with rifampicin and demonstrated that it had no effect on carotenoid synthesis, implying that prokaryotic (or plastid) transcription is unnecessary for this process. [Ellis (1977) points out that rifampicin has only been shown to prevent the synthesis of plastid rRNA and allows the synthesis of Rubisco subunits and cannot therefore be considered a specific inhibitor of plastid protein synthesis, casting doubt on the conclusions of Sirevåg and Levine (1973).] These authors also tested spectinomycin, an inhibitor of 70S ribosomes and found that it reduced carotogenesis in *Chlamydomonas*. Camara (1984) investigated carotenoid sythesis in *Triticum* leaves which were deficient in plastid ribosomes, and found that synthesis still took place. Treatment with chloramphenicol and lincomycin (both inhibitors of 70s ribosomes) had no effect. Baker *et al.* (1985) found that tomato ripening was inhibited by cycloheximide but not chloramphenicol, thus corroborating the results of Camara

(1984). The overall results of the antibiotic studies imply that plastid protein synthesis is unnecessary for the process of carotogenesis, whereas cytoplasmic protein synthesis is necessary.

Carde *et al.* (1988) studied protein synthesis in *C.annuum* fruit chromoplasts. They showed disappearance of ribosomal particles (monitored electron micrographically) concomitant with a decrease, throughout ripening, of 16S and 23S plastid rRNA. Fully differentiated ripe fruit chromoplasts did not contain rRNAs or ribosomes. It was suggested tha the complete disappearance of ribosomal structures is a sign of complete transformation from chloroplast to chromoplast. This evidence supports the concept that carotenoid enzymes, and other proteins active in the ripe fruit chromoplast, are encoded on nuclear genes, translated on cytoplasmic ribosomes, and integrated into the plastid by a post-translational transportation mechanism. This developmentally induced passivity of the chromoplast genome is not accompanied by alteration in the plastome DNA, which has been shown to be similar to chloroplast (Gounaris *et al.*, 1986).

Further indication that ripening is a nuclear controlled process comes from the study of ripening mutations, which prove to be inherited in a Mendelian fashion. If the mutations were encoded by plastid DNA they would be inherited maternally or in an uneven bi-parental fashion. Tomato mutants Never Ripe

(NR) and Ripening Inhibited (RIN) are probably the most studied of ripening mutants. NR is a mutation located on chromosome 9 and exhibits reduced ethylene, low lycopene and slow softening, becoming a pale orange colour in its ripest state. RIN is located on chromosome 5 and exhibits no detectable ethylene, no lycopene and no softening, becoming a pale yellow colour in its ripest state. Both mutations are inherited in a Mendelian fashion (Simpson *et al.*, 1977). Ripening mutants of pepper were investigated by Laborde and Spur (1973), who identified nuclear genes responsible for the breakdown of the photosynthetic apparatus and the deposition of carotenoids. The authors suggested alleles were as follows:

Y+: allows formation of the red pigment.

Y: (recessive) blocks formation of the red pigment.

Cl+: allows disintegration of the chloroplast grana.

Cl: (recessive) blocks disintegration of the grana.

Wild type peppers were described as Y+Y+ and a yellow mutant as YY Cl+Cl+, the mutation being inherited in a Mendelian fashion. More recent work on the inheritance of ripening mutations in pepper (Hurtado-Hernandez and Smith, 1985) points to the existence of three pairs of ripening genes, the functions of which are outlined below:

Y+ and Y: production and non-production of the red pigment.

C_1+ and C_1: no inhibition of pigment production and inhibition of pigment production.

C_2+ and C_2: no inhibition and strong inhibition of pigment production.

[Those genes bearing a (+) are dominant.]

Their deductions were based on the cross of a homozygous wild type red fruiting variety with a homozygous recessive albino, giving an F1 generation, which, when back-crossed to the albino, gave eight different phenotypes. The earlier observations by Laborde and Spur (1973) appeared to be based on a limited number of phenotypes, giving a misleading conclusion. These experiments emphasize the Mendelian nature of inheritance of fruit ripening mutations, and hence imply chromosomal rather than plastidic inheritance.

The final evidence that ripening is primarily controlled from the cell nucleus rather than an organellar compartment comes from comparisons of the *in vitro* translation profiles of total and polyadenylated RNA, from ripe and unripe fruit. The total and poly(A)+ RNAs from fruit at different stages was compared and the difference between ripe and unripe shown to lie in the poly(A)+ fraction, generated from the nucleus (tomato, Rattapanone *et al.*, 1978; avocado, Christoffersen *et al.*, 1982). Plastid RNAs have been shown to lack poly A tails.

Suggestions of how nuclear proteins may control a plastid developmental process such as ripening have been drawn from Ellis (1977). The nucleus and cytosol produce polypeptides which are translocated into organellar compartments by means of a leading transit peptide which is cleaved off during the passage through the organelle membrane. Keegstra and Bauerle

(1988) have recently demonstrated the existence of further consensus sequences at the amino end of the protein, which direct the polypeptide more precisely within the plastid, to its destination. Additional sequences exist to transport proteins to the thylakoid membranes and plastid lumen. The conformation of the protein to be imported has been shown to be important, more so than the primary sequence which is not particularly well conserved (Ellis and Robinson, 1987; Meyer, 1988). Proteins to be imported have an unfolded conformation that is maintained by a cytoplasmic protein component(s), 'trigger factor' the absence or presence of which provides another possible method for regulation. Recently a receptor protein has been identified in contact sites between the outer and inner chloroplast envelope that participates in protein import into the chloroplast (Pain *et al.* 1988). Thus cytoplasmic proteins can be precisely placed in the plastid, and once there carry out their function, whether this be structural, enzymic, as in the case of the carotogenesis enzymes, or regulatory, affecting the plastid protein synthesis apparatus in some way.

1.1.08 Plastid and nuclear co-operation.

It is well known that the nucleus and cytosol provide the plastid with most of its polypeptides via the organellar envelope. Once inside, these proteins affect metabolism and possibly plastid gene expression. For example, a nuclear

mutation of *Chlamydomonas reinhardtii* has been shown to cause reduced accumulation of plastid encoded polypeptides, by affecting the translation or stability of the plastid gene product (Kuchka *et al.* 1988). It is believed that most plastid genes are constitutively expressed, and are controlled post-transcriptionally, possibly by nuclear factors (Deng and Gruissem, 1988).

Interest has also been expressed in the possibility of plastid-derived factors which influence cytosolic protein synthesis. Treatment with the herbicide Norflurazon inhibits carotenoid synthesis and leads to chloroplasts bleached through photo-oxidation of chlorophyll, which mimics the effect of an Albina mutation of barley. In both the mutant and the herbicide treated plants, functional chloroplasts have been eliminated, and in both, transcription of the nuclear encoded light harvesting chlorophyll a/b protein (LHCP) is inhibited (Batschauer *et al.*, 1986). The inhibition seems to be specific for the LHCP and other nuclear genes are not affected; this led the authors to conclude that plastid derived factors are necessary for continuous light dependent transcription of the nuclear gene encoding the LHCP. A nuclear mutation of barley which causes plastids deficient in ribosomes, pigments and protein synthesis also causes a lack of many nuclear encoded plastid proteins (Bradbeer *et al.*, 1979). Some enzymes are present in very small amounts and it is possible to analyse their activity, which shows that there

is nothing wrong with the structural genes themselves or their transcription mechanism. It is the level of transcription which has been greatly reduced, and if the chloroplast DNA is damaged, transcription of these nuclear plastid genes stops altogether. The authors suggest that a non-protein plastidic factor, for example an RNA, acts to de-repress cytoplasmic translation of plastid protein genes. Bradbeer *et al.*, (1979) suggested that a plastid encoded RNA which was translocated into the cytoplasm may act as a tRNA during cytoplasmic translation. Its presence would therefore enhance translation, and its absence reduce it. Miller McCrea and Hershberger (1978) showed that chloroplast DNA contains a number of cistrons which do not hybridize to chloroplast RNA, but hybridize instead to RNA from the cytoplasm, also suggesting that the plastome codes for RNAs which may be used during cytoplasmic translation. Further evidence for plastid control of nuclear gene expression is presented in a review by Borner (1986), which presents numerous examples of the necessity for functional plastids before nuclear encoded plastid gene products can be expressed normally. For example, in barley and *Euglena*, deficiencies in plastid structure or DNA resulted in greatly reduced amounts of nuclear encoded plastid proteins, while expression of other nuclear genes was unaffected. In both *Zea mays* and mustard, plastid deficiency resulting from herbicide treatment or mutation caused repression of LHCP and Rubisco small subunit gene expression. It was shown that light regulated genes are only expressed

where there are functional plastids and are not expressed where there are not. In tuber tissue for example, should the tuber start to 'green' and develop functional plastids, it then becomes possible to express the nuclear encoded plastid genes. It is thought that functional plastids produce a factor which targets the promoter region of nuclear encoded plastid genes and enhances the transcription of these genes. Plastome RNA acting as a tRNA during cytoplasmic translation is a possible method of enhancement. Lack of a plastid factor could also enhance cytoplasmic translation, by a de-repression mechanism.

1.1.09 Ripening as a developmental process.

Evidence that ripening is an active developmental process involving differential gene expression, rather than a degradative senescence involving a breakdown of 'organizational resistance' and the release of hydrolytic enzymes, has come from the study of ripening fruit at the levels of transcription and translation. By looking at the proteins, the RNA and finally the DNA, it has been possible to demonstrate the developmental nature of ripening.

Polyribosomes were shown to persist throughout ripening of the tomato (Speirs *et al.*, 1984), implying that protein synthesis occurs during this process. *In vivo* labelling of tomato fruit pericarp showed that the proteins were labelled during the

ripening process and that the protein complement changed as the fruit progressed from unripe to ripe (Baker *et al.*, 1985). The proteins of purified tomato plastids from ripe and unripe fruit were submitted to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and shown to differ (Bathgate *et al.*, 1985). Three classes of protein were identified; those that were present in both ripe and unripe fruit; those present in unripe fruit only; and those present in ripe fruit only. The changes in the mRNA population were studied using the technique of *in vitro* translation in a rabbit reticulocyte system or a wheatgerm translation system (Rattanapanone *et al.*, 1978). The translation product profiles of ripe and unripe fruit were different, reflecting differences in the mRNA populations. There were three classes of product corresponding to the three protein classes already identified. A cDNA library was made from ripe fruit mRNA and screened with labelled cDNA from either red or green fruit (Grierson *et al.*, 1985). Those clones which were differentially complementary to the red probe were selected as ripening specific. Cross-hybridization of the clones to each other was used to group the clones and the largest clone in each group was taken to be a typical member of that group. Hybridizations to Northern blots of ripe fruit RNA indicated that many of the clones were the same size as the message, i.e. the clones were full size. Hybrid release experiments allowed study of the protein products of the clones and these could then be matched to the *in vitro* translation products. By performing hybridization

experiments with RNA from different organs, or different stages of fruit development, the authors were able to demonstrate the organ specificity and ripening specificity of some of the clones. In other words they showed that the expression of the genes was confined to a particular spatial and temporal programme. A clone for the tomato ripening softening enzyme, polygalacturonase was identified using immunoprecipitation, and also by comparing clone sequences to the N-terminal amino acid sequence of purified PG. Using this clone it was possible to examine the incidence of PG mRNA during normal and mutant tomato ripening. The Ripening Inhibited (RIN) mutant of tomato was found to have no detectable PG which may explain its lack of softening. Normal fruit show increasing levels of PG as ripening proceeds. Treatment with ethylene resulted in the measurable expression of ripening specific genes and was demonstrated using *in vitro* translation and hybridization of RNAs to the ripening specific clone.

A similar story can be told for the avocado (Christoffersen *et al.*, 1982). Protein and RNA populations were shown to be labelled throughout ripening and components of these populations were shown to alter during the process. A cDNA library was created from ripe fruit mRNA and those clones which hybridized preferentially to ripe fruit cDNA rather than unripe were designated ripening specific. Cellulase is the enzyme responsible for the softening of avocados and a

cellulase clone was identified by hybrid release translation and co-migration with purified cellulase. Using hybridization of the labelled clone to RNA it was possible to follow the changes in the RNA population as the fruit ripened. It was also possible to demonstrate the organ-specific and stage-specific nature of cellulase gene expression (Christoffersen *et al.*, 1984).

1.1.10 Plant gene expression.

Ripening can be described as a developmental process involving differential gene expression. Sets of genes are switched off and other sets are switched on, and some genes show variation in the level of their expression as ripening progresses. Other genes show no alteration of expression at all. In order to hazard a guess at how such differential gene expression is possible, it is instructive to look at models of plant gene expression developed for different plant systems.

An example of how a particular gene may be expressed in preference to others is found in the rRNA genes of wheat. Wheat rRNA genes are contained in all the nucleolar regions of the wheat chromosomes but only expressed from one such region (the dominant nucleolus). The dominant nucleolus has been found to have the most so-called 'enhancer' sequences, which is the reason it becomes dominant. The enhancer sequences are beleived to compete for a binding factor, probably a protein,

which enhances transcription by helping the binding or formation of the transcription complex, or by helping the movement of the complex, or by increasing the concentration of the complex (Grierson and Covey, 1984).

An investigation into the seed storage proteins of soybean indicated that they represent a highly regulated gene set and are temporally and spatially differentially expressed (Goldberg, 1986; for a review, see Goldberg *et al.*, 1989). A model was proposed to explain this expression which suggested that each structural gene has a number of *cis*-acting elements which act as transcriptional enhancers and maybe repressors. Each element responds to a specific *trans*-acting factor, which is only present in particular situations, for example in a particular organ or at a particular stage of development. This would make it possible for a gene to be expressed in a temporally and spatially defined way. Soy bean lectin genes were fragmented and incubated with embryo nuclear protein extracts, and then separated by gel electrophoresis. Retardation of the fragments was shown to occur indicating that proteins in the extract had bound to the DNA. Further experiments showed that the gene fragments would bind only to embryo nuclear protein where expression was taking place and not to nuclear protein from other parts of the plant. Deletion studies indicated that the binding sequence was -100 to -400 base pairs upstream of the start of the structural gene. A possible *trans*-acting factor was therefore identified. The

soybean β-conglycinin seed storage protein gene (Chen *et al.*, 1988), possesses a *cis*-acting regulatory element which confers spatial and temporal regulation of gene expression as does the soybean leghaemoglobin *lbc*$_3$ gene (Jensen *et al.*, 1988).

Some plant genes may be expressed in a number of tissues but to different levels. In an attempt to explain the expression of α-amylase genes in wheat, which are expressed in the aleurone cells of the grain in response to giberellin, Baulcombe *et al.* (1986) proposed three possible models of gene expression. The models are not mutually exclusive, but are rather used to explain a variety of possible mechanisms. (i) The first model proposed that multiple genes exist for a particular set of isoenzymes, and that different modes of expression are possible using the different genes. Eukaryotic genes believed to operate like this include glutamine synthase in *Phaseolus*, and maize phosphoenolpyruvate carboxylase.
(ii) The second model consists of a single structural gene with different promoters which are used alternatively to produce different 5' exons which are then spliced to form the mature RNA. Genes believed to have this kind of structure include mouse α-amylase which is expressed differently in the liver, pancreas and salivary gland, *Drosophila* alchohol dehydrogenase and mammalian immunoglobulin genes. (iii) The third model has a single structural gene and a single promoter but several *cis*-acting regulators which is approximately what was proposed by Goldberg (1986) for soybean lectin genes.

Different cell types would produce different *trans*-acting elements and therefore different expression profiles. Genes believed to have this type of expression include the chicken lysozyme gene (expressed in macrophage cells and the oviduct) and wheat carboxypeptidase genes. As mentioned briefly in section 1.1.03, a 5' flanking region of an ethylene responsive gene has been identified that binds a nuclear protein in correlation with its level of expression during ripening. The model proposed for this ripening gene seems to be that proposed by Goldberg (1986) for soybean lectin genes, i.e. a *cis* element that acts as an inducer of transcription when bound by a factor, or factors, from *trans* elements some distance away (Diekman and Fischer, 1988).

As well as transcriptional regulation there is evidence that a number of plant genes are regulated post-transcriptionally. Light induced chloroplast formation in higher plants has been found to involve numerous factors including phytochrome, the conversion of protochlorophyllide to chlorophyllide, and changes in chromatin structure (Apel *et al.*, 1986). Also implicated is a plastid derived factor that moderates the expression of nuclear encoded plastid genes. Post-transcriptional control has been implicated in the expression of one plastid protein, the P700 chlorophyll a protein, whose genes have been localized to the plastid DNA. This protein is constitutively expressed in both light and dark conditions, but accumulates only in the light. The protein is stabilized

by chlorophyll which is present only in the light; in the dark the P700 apo-protein is rapidly destroyed. Another possible method of post-transcriptional control may lie in the binding of ribonucleoproteins to the message (Richter, 1988). Some mRNPs bind ubiquitously to all mRNAs, e.g. those proteins that bind the CAP site and the poly(A) tail. Other proteins bind specific sequences and may regulate translation.

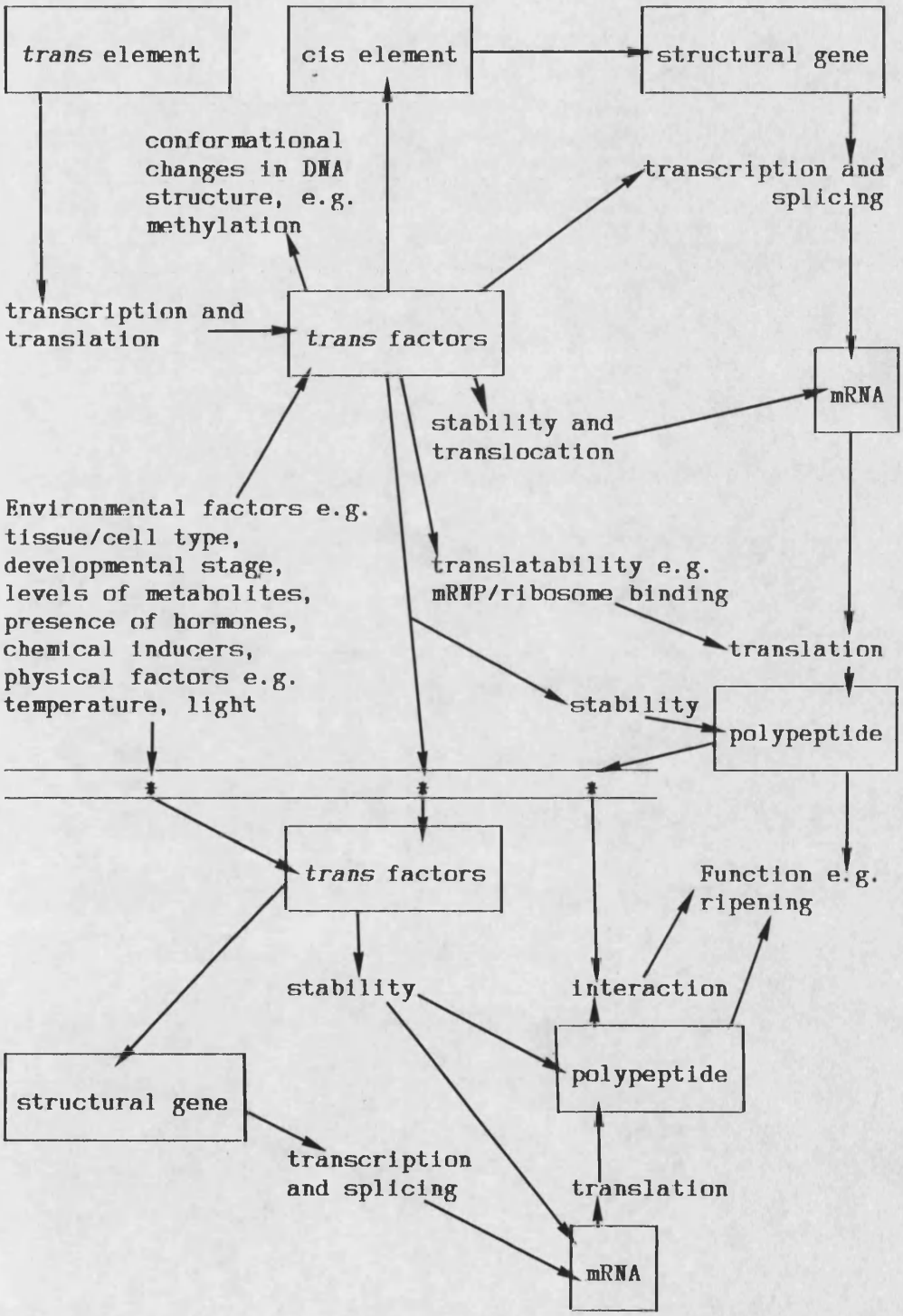
Post-transcriptional regulation of plastid gene expression was studied by Deng and Gruissem (1988) who looked at the expression of a number of mRNAs in non-photosynthetic plastids. There was found to be constitutive expression of plastid RNA, which was found to be post-transcriptionally controlled by preferential translation or message stability. Plastid RNAs are transcribed, and often translated, as polycistronic units, and the type and amount of processing of these units may well have an effect on expression (Barkan, 1988). The stability of plastid mRNA is increased by the presence of 3' inverted repeated sequences (Stern and Gruissem, 1987). Environmental factors are thought to affect the structures of the 3' inverted repeats, so altering plastid gene expression. A diurnal pattern of expression has been observed for photosynthetic genes of early green tomatoes which is presumably imposed by the environmental day/night cycle (Piechulla and Gruissem, 1987). Many RNAs e.g. histone RNA, granulocyte monocyte colony stimulating factor RNA, the transferrin receptor RNA and *Drosophila*'s *c-fos* RNA are shown

to be stabilized by the presence of 3' structures, such as the polyadenylated region, which is believed to protect the RNA from degradation by nucleases. RNAs are preferentially degraded in particular situations (Hunt, 1988). The regulation of gene expression by specific RNA degradation or maintenance is therefore an established hypothesis.

1.1.11 Summary.

Figure 4 summarizes the ideas put forward in this introduction. Ripening is viewed as a 'function', the result of interaction between external factors, nuclear *cis* and *trans* factors, and plastid *cis* and *trans* factors. The diagram is necessarily simplified and does not show many of the possible ramifications of the system. It does show the flow of communication between the plastid and nucleus, and at least partially demonstrates the continuously alterable network, controlling the developmental state of the plant cell. The aspirations of the work will be explored more fully in the discussions, so it will suffice to say it was with the aim of understanding some of this subtlety that this project was undertaken.

Figure 4. Possible modes of gene expression regulation in a higher plant cell.



____ plastid membrane

‡ translocation across the plastid membrane

Chapter 2. Pigmentation, ultrastructure and protein complement of *C.annuum* fruit during ripening.

2.1.00 Introduction.

The main aim of this first group of experiments was to investigate the changes taking place in ripening fruit as evidence for changes in gene expression. General studies of change in the ripening fruit were carried out, including a brief look at pigment change, an electron microscopic study of the plastids of ripening fruit, and various studies on the protein population, carried out with the aim of identifying particular species related to ripening. Standard procedures were used with varying degrees of success. The background to the work is described in the General Introduction in sections 1.1.04 (ultrastructural changes during ripening); 1.1.05 and 1.1.06 (changes in metabolism and pigmentation); and section 1.1.09 (ripening as a developmental process, involving changes in protein). These experiments were included to provide an experimental rather than an assumed basis for further work, for the sake of completeness, for interest, and to correlate with later work at more fundamental levels of gene expression.

2.2.00 Materials and Methods.

2.2.01 Plant material.

Plants of *Capsicum Annuum* var. Bellboy, Lito and Golden Star were grown from seed under normal glasshouse conditions. The plants were kept at a temperature of about 25°C, and supplemented with extra lighting if necessary. No attempt was made to grow the plants under sterile conditions and as a result of pest attacks the plants were regularly treated with insecticides. Fruits were selected on the basis of their appearance, their colour being used as a guide to their state of ripeness. Any fruit tissue found to be at an incorrect stage was discarded. Fruits were picked with gloved hands and washed in distilled water and ethanol prior to any experimental work.

2.2.02 Chemicals.

The chemicals used for the fixation and staining of pepper tissue were the purest available, of EM grade, from BDH, Sigma and Aldrich. The acrylamide and bis-acrylamide were electrophoresis grade from Sigma.

2.2.03 Photography.

The photographs of pepper fruit were taken in a studio using a Hasselblad 50c camera and a Vericolor 'L' 120 film.

2.2.04 Pigment extraction.

Pepper pericarp tissue was ground under liquid nitrogen to a fine powder and extracted in a ratio of 1g per ml in 80% acetone. The resultant coloured solution was filtered through Whatmann 3MM paper and stored covered at -20°C until scanned. The pigment solutions were scanned in quartz cuvettes in a Unicam SP 1800 spectrophotometer against an 80% acetone blank. The spectrophotometer scanned through wavelengths from 750nm to 180nm and the relative absorbances of the test solution at each wavelength were plotted by a chart recorder.

2.2.05 Ultrastructural study.

Pepper pericarp tissue was cut into $0.5mm^3$ pieces and incubated for one hour in 2.5% glutaraldehyde in 0.05M sodium cacodylate buffer, pH 7.2, at 4°C. The tissue pieces were then transferred to fresh 0.05M cacodylate buffer, pH 7.2, and incubated overnight at 4°C. The samples were transferred to 1% osmium tetroxide in cacodylate buffer and left at room temperature in a fume hood for 1 hour. The samples were washed twice in distilled water and then block stained in 1% tannic acid in water for approximately 1 hour at room temperature. The tissue was dehydrated by washing in increasing percentages of acetone, 30%, 50% and 70% for two lots of 10 minutes each; and finally in 100% acetone for four lots of 15 minutes. The pieces were then put into a 50:50 mix of resin and 100%

acetone and could be stored in the freezer at this point. The tissue was transferred to a 3:1 resin:acetone mixture (the resin was Emix from Emscope) and stirred slowly on a rotating stirrer at room temperature overnight. The pieces were then switched to 100% resin and left on the stirrer for a further 6 hours; and then left in fresh 100% resin overnight. The next day the resin was changed yet again, and the tissue left stirring overnight in fresh resin. The samples were finally 'blocked up' in rubber moulds containing labelled cavities and dried in a bench oven at 50°C for 2 days. Sections were cut on a Reichert OMU3 ultramicrotome. The sections were viewed in a JEOL 100 cx transmission electron microscope.

2.206 Plastid purification.

All procedures were carried out at 4°C.

Washed and dried fruit tissue was weighed and homogenized in buffer A (0.25M Tris-HCl, pH 7.6; 0.5M sucrose; 0.01M $MgCl_2$; 0.04M β-mercaptoethanol) at a concentration of 1g per ml. The homogenate was filtered through three layers of muslin into bench centrifuge tubes. The tubes were centrifuged at 2,000*g* in a bench centrifuge to spin down cell debris (there was often no pellet at this stage) and the supernatant transferred to fresh tubes and centrifuged at 4,000g to bring down the plastid pellet. The pellet was resuspended in a suitable volume of buffer B (0.05M Tris-HCl, pH 8.0; 0.01M $MgCl_2$; 0.04M β-mercaptoethanol) and layered onto stabilized sucrose gradients

prepared the previous day. The gradients consisted of four steps from 20% w/v sucrose to 55% w/v sucrose, made up in buffer B, and left to stabilize overnight at 4°C. The loaded gradients were centrifuged at 90,000*g* (26,000 r.p.m. in an SW28 Beckman ultracentrifuge rotor) for 35 minutes at 4°C.

Two major bands were usually produced, the upper containing broken plastids and the lower containing intact plastids, demonstrated by phase-contrast microscopy of the plastids (Arundel, 1984). Intact plastids have a 'halo' around them. The lower band was taken off with a pasteur pipette and put with an equal volume of buffer B. The plastids were then centrifuged at top speed in a bench centrifuge to pellet the purified plastids. The pellet was resuspended in the minimum volume of buffer B and represented the plastid preparation. Treatment of the plastids depended on the experiment they were to be used for but they were usually lysed (with 5% w/v SDS or Triton-X100) and stored at -20°C.

2.2.07 Dissociating gel electrophoresis.

The method used was that described by Hames (1981), which is based on the method originally published by Laemmli (1970). A 10% polyacrylamide gel was made be mixing 10ml of 30% acrylamide/0.8% bis-acrylamide, 0.3ml of 10% SDS, 1.5ml of freshly made 1.5% ammonium persulphate, 14.45ml of distilled water and 3.75ml of resolving buffer (3M Tris-HCl, pH 8.8). The

mixture was de-gassed to remove oxygen which could interfere with polymerization, and 15µl of TEMED (tetramethylethylene diamine) the polymerization catalyst were added and mixed by gentle swirling. The mixture was poured between two glass plates of an Atto electrophoresis system, and overlaid with butan-1-ol to ensure an even set. When the resolving gel had set a 3.75% stacking mixture was made up by mixing 2.5ml of 30% acrylamide/0.8% bis-acrylamide, 0.2ml of 10% SDS, 1ml of freshly made up 1.5% ammonium persulphate, 11.3ml of distilled water and 5ml of stacking buffer (0.5M Tris-HCl, pH 6.8). The mixture was de-gassed and 15µl of TEMED added as before. The stacking mixture was layered onto the rinsed resolving gel and a comb inserted. The gel was left to set for about an hour.

Samples were prepared by boiling for 3 minutes with an equal volume of sample buffer (0.125M Tris-HCl, pH 6.8; 4% w/v SDS; 10% β-mercaptoethanol; 20% w/v sucrose; 0.004% bromophenol blue) and then centrifuging at top speed in a microfuge for 3 minutes to pellet insoluble matter that could cause streaking on the gel.

Samples were loaded into the wells and run in running buffer (0.025M Tris-HCl, pH 8.3; 0.192M glycine; 0.1% SDS) at 150v for about 6 hours or until the bromophenol blue dye had reached the foot of the gel.

Gels were stained in Coomassie blue (0.1% Coomassie blue; 40% methanol; 15% acetic acid) and destained in 12% methanol/10% acetic acid. The destained gels were then analysed by scanning on a Joyce Loebl gel scanner or by eye. The gels were dried down on an Atto gel drier for storage.

2.2.08 Isoelectric focusing.

(i) Running the gel.

The isoelectric focusing of purified plastid proteins (lysed with Triton rather than SDS) was done using the Phastgel system manufactured by Pharmacia. Prepared gels (pH gradient 3-10) were pre-run for 75 volthours to establish a gradient and then the samples were applied centrally. The samples were allowed to run for 425 volthours to ensure that all the proteins had reached their isoelectric points. IEF gels were stained using the protocol recommended by Pharmacia.

(ii) Staining the gel.

The gel was fixed for 5 min at 20°C in 20% w/v TCA. It was washed for 2 min at 20°C in wash 1 (50% v/v ethanol; 10% v/v acetic acid) and washed for 2 min and then 4 min at 50°C in wash 2 (10% v/v ethanol; 5% v/v acetic acid). The gel was treated with sensitizer (8.3% w/v glutaraldehyde) at 50°C for 6 min. This was followed by two washes of wash 2 at 50°C for 3 and 5 min respectively, and a further two washes with wash 3 (distilled water) at 50°C for 2 min per wash. The gel was

then stained with 0.5% w/v silver nitrate in water at 40°C for 10 min, and washed twice more with water at 30°C for two lots of 5 min. Developer (0.04% w/v formaldehyde; 2.5 w/v Na_2CO_3 was added at 30°C for 0.5 and then 4 min. Gels were stored wet in acetic acid (7% v/v), dried by leaving at room temperature for a few days or dehydrated using a hair-dryer.

2.2.09 Two-dimensional gel electrophoresis.

(i) Sample preparation and running the first dimension.

The proteins were run on IEF gels supplied by Pharmacia as described in section 2.2.08 (i), pH gradient 3-10. An equivolume of 2D sample buffer (2.88g urea; 1.0ml Triton; 0.25% v/v of 20% w/v DTT; 0.1ml Pharmalite 3-10; to 5ml with distilled water) was added to the sample which was then left for 1 hour on ice, and centrifuged at top speed in a microfuge for 3 min. The supernatant was applied directly to the gel. Ampholyte is present in the sample buffer to aid solubility and prevent streaking in the 2nd dimension gel. The samples were applied centrally and in lanes rather than across the whole gel.

(ii) Treatment of gel strips.

The parts of the gel that the electrodes were resting on were cut away using scissors, and strips containing the samples were cut from the gel. The lanes were located by identifying the indentations made by the sample applicator. Gel strips were appropriately marked on the gel bond backing of the gel naming

the anode and cathode and the sample type. Strips were approximately 3mm by 32mm. The strips were incubated in equilibration buffer 1 for 2.5 min (0.112M Tris base; 0.112M acetic acid; 1% w/v DTT; 2.5% w/v SDS), and then equilibrated for another 2.5 min in equilibration buffer 2 (as buffer 1 but with only 1% w/v DTT; 0.26M iodo-acetamide; 0.1% w/v bromophenol blue; adjusted to pH 6.4 with NaOH). Iodo-acetamide binds excess DTT which can interfere with silver staining. This equilibration step serves to wash out much of the urea and ampholytes from the gel and equilibrate the sample for the SDS-PAGE separation. Some protein may also wash out of the gel at this stage.

(iii) Running the 2nd dimension.

The equilibrated strip was placed gel side down on the stacking zone of a Pharmacia PhastGel Gradient 10-15 gel, as close to the buffer strip as possible, so as to allow the sample components to stack. Pharmacia molecular weight marker proteins were applied alongside the strip, adjacent to the cathodic end of the strip, where they would interfere least with the sample proteins. The gel was run at 15°C, 250v for the first 5vhours, and then the strip was removed to prevent electrical field disturbances. The gel was run for a further 60vhours at 250v, or until the dye reached the anodic buffer strip.

(iv) Staining the 2D gel.

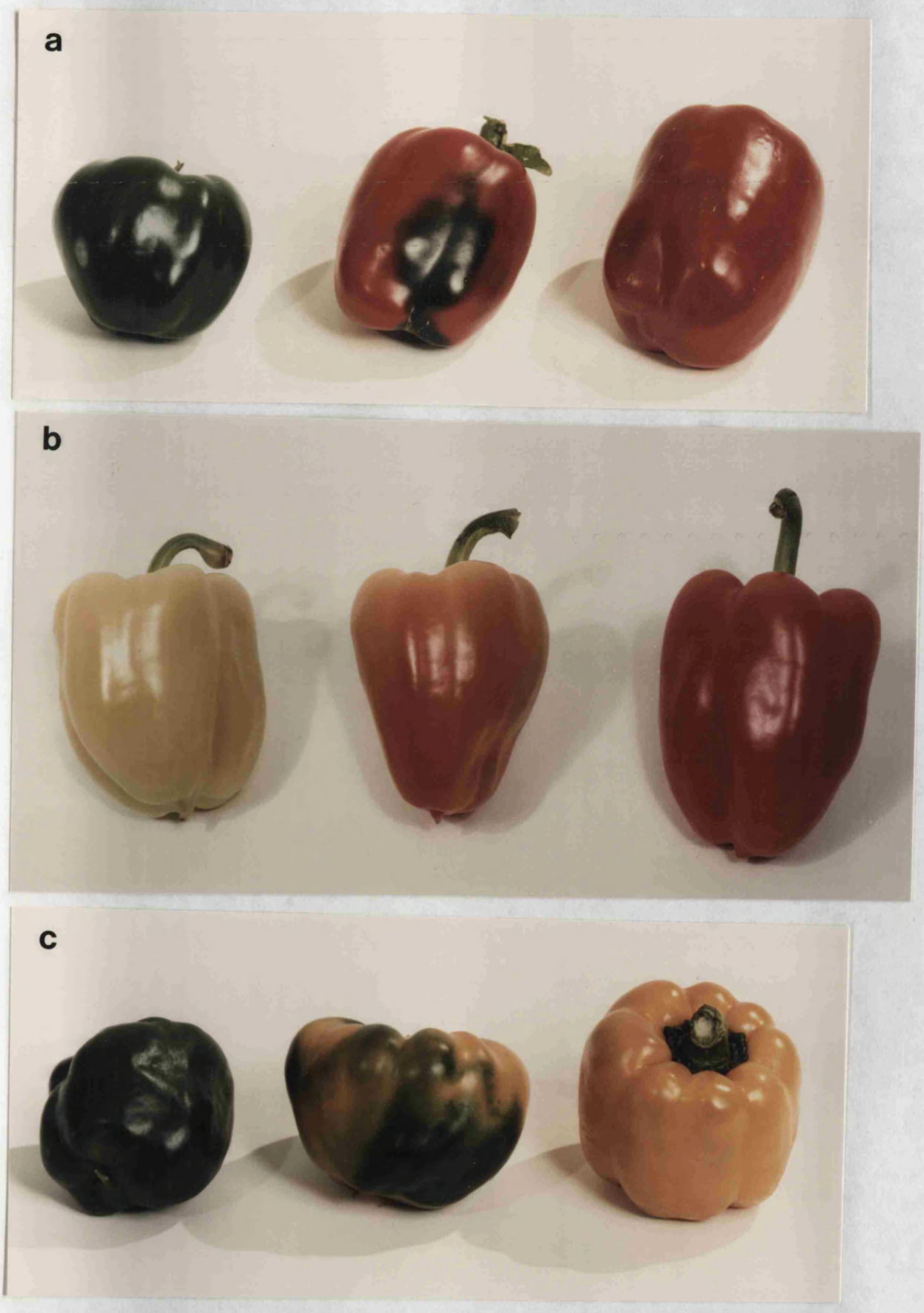
The gel was stained as for IEF gels except there was no fixation step in 20% w/v TCA, the gels spent 13 min at 40°C in 0.25% w/v silver nitrate, and they were finally treated with a preserving solution (10% v/v acetic acid; 5% v/v glycerol) at 50°C for 3 min after the stop bath step. Gels were stored as described for IEF gels in section 2.2.08 (ii).

2.3.00 Results and Discussion.

2.3.01 Pigments.

During ripening *C.annuum* Bellboy fruits go from leaf green, through a 'breaker' stage of a patchy red and green appearance, to crimson (Figure 1a). The visual effect can be followed spectrographically by extracting the pigment from fruit at different stages into acetone and scanning through various wavelengths to obtain absorbance spectra. This was done for Bellboy fruit (Figure 1d), and the ripe fruit showed increased absorbance in the wavelengths 410-470nm and loss of a peak of absorbance at 668nm (loss of chlorophyll). This correlates well with results reported in the literature (Wrench *et al.*, 1987). Camara and Monéger (1978) showed that fully expanded green *Capsicum* fruit had the same absorption spectra as leaves (although with approximately 40x less chlorophyll; (Davies *et al.*, 1970). They showed the disappearance of chlorophylls *a* and *b* and the simultaneous appearance of ripe fruit associated carotenoids including cryptocapsin, capsanthin, violaxanthin, zeaxanthin, antheroxanthin and capsorubin. The pigments responsible for the red colour of *C.annuum* ripe fruit were found to be capsanthin and capsorubin. Candela *et al.* (1984) performed thin layer chromatography on extracts of ripe *Capsicum* fruits and identified the same species as Camara and Monéger (1978). Lito fruit progresses from 'white' (or very pale green) through an orange stage to a red fruit similar in

Figure 1. Pigment changes in ripening fruit.



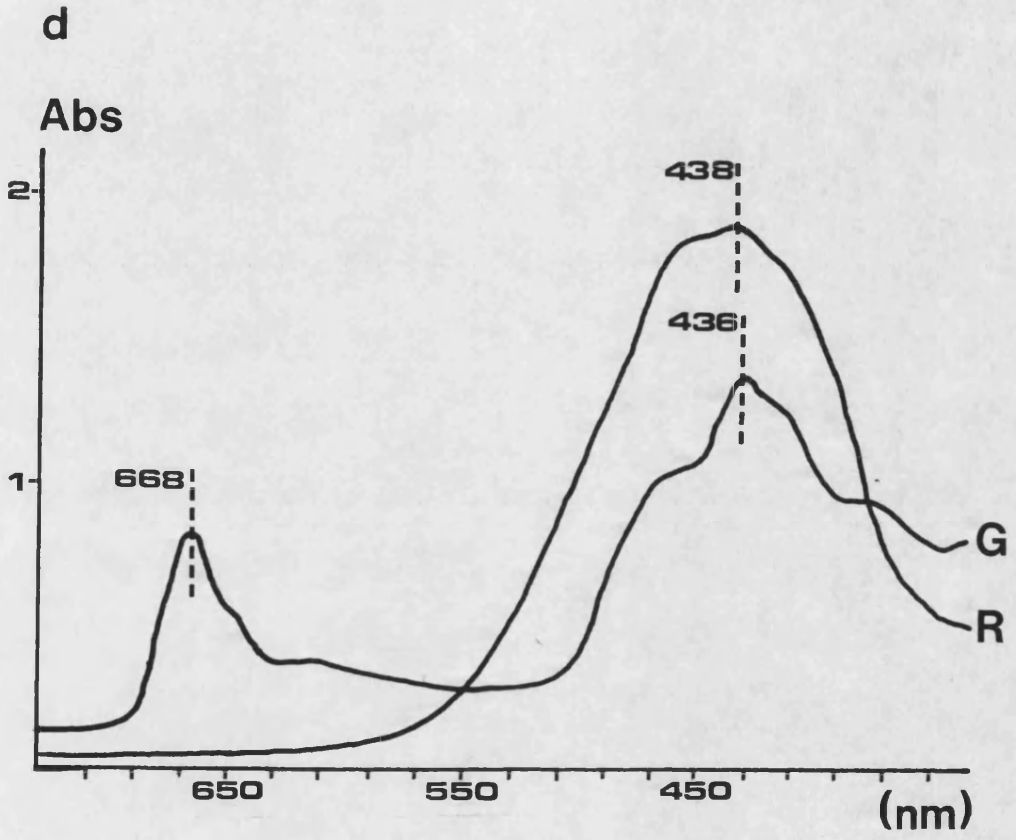


Figure 1. Parts a, b and c show unripe, intermediate and ripe fruits of *Capsicum annuum* var. Bellboy, Lito and Golden Star respectively. Part d shows the absorbances at different wavelengths of extracts of unripe and ripe fruits of Bellboy. The fruit tissue was ground to a fine powder under liquid nitrogen and extracted at a concentration of 1g/ml in 80% v/v acetone. This coloured extract was filtered and scanned in a quartz cuvette against an 80% acetone blank in a spectrophotometer (as described in Materials and methods, section 2.2.04). The x-axis represents wavelengths from 750 to 180nm and the y-axis represents the absorbance of each extract at that wavelength. The method was taken from Arundel (1984).

appearance to Bellboy (Figure 1b). Golden Star pepper fruit (Figure 1c) pass from a green fruit very similar in appearance to unripe Bellboy, through a spectacular marbled green and yellow intermediate stage, to a final deep golden yellow. A study of the carotenoids of different types of ripe pepper fruit was performed by Davies *et al.* (1970). It was found that yellow fruit had an absorption spectrum very similar to that of unripe green fruit except in the lower wavelengths. Yellow fruit were shown to contain about 5x more total carotenoid than unripe fruit, so carotenoid synthesis does take place (ripe red fruit contain 100x the carotenoid content of unripe fruit). The only carotenoids lacking from the yellow fruit are the red pigments capsanthin and capsorubin.

2.3.02 Ultrastructure.

The types of fruit studied were Lito and Golden Star, in an attempt to look for differences from and similarities to Bellboy, as well as follow the changes associated with ripening in those fruit. Ultrastructural studies on the normal ripening of *C.annuum* sweet bell pepper have been performed several times; the work used as a reference was that by Arundel (1984) which was performed in our laboratory on *C.annuum* var. Bellboy, using a similar fixation technique and the same electron microscope. Four types of chromoplast have been identified in ripe fruit according to the appearance of the thylakoid plexus. These are globular, tubular, membranous and crystalline

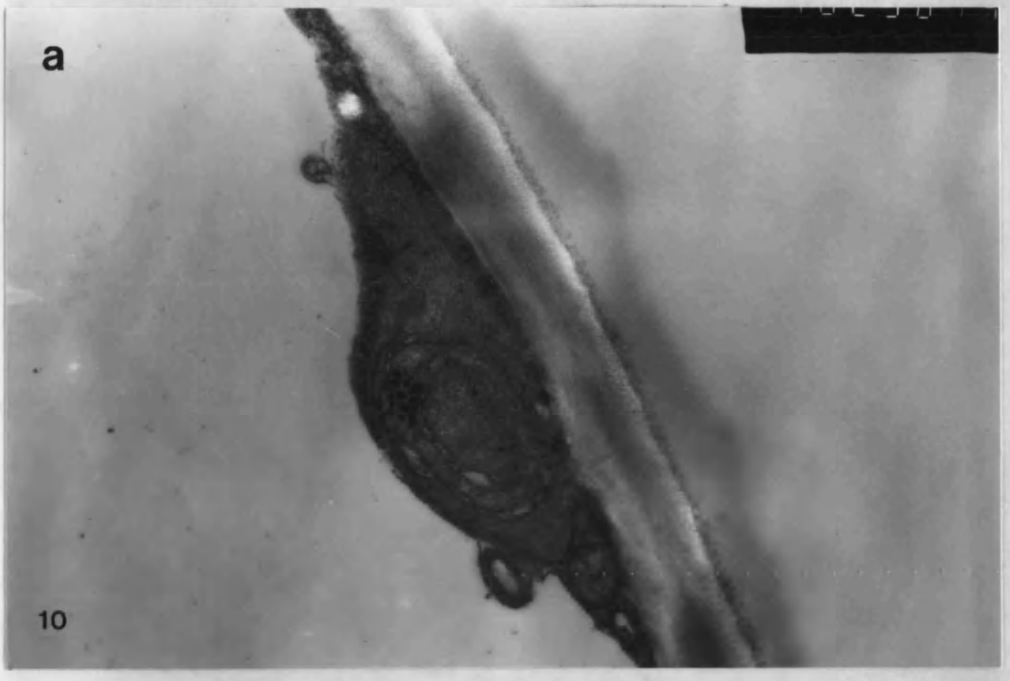
(Arundel, 1984; Whatley and Whatley, 1987). *Capsicum* chromoplasts are designated as tubular, due to the presence of large numbers of tubules. Unripe Bellboy fruits have a granal organization typical of chloroplasts. Starch granules may be present, as bodies with low electron density, and there are a few lipid containing osmiophilic globules (Arundel, 1984). As the fruit ripens the starch granules become smaller, the number of globules increases and the membrane structure becomes less well organized, although the grana may remain relatively intact. In the ripe fruit there is wide variation in the ultrastructural state of the cells. There are usually many fibrils (or tubules) with osmiophilic swellings, and osmiophilic globules, which possibly originate from the fibrils. The thylakoids become abnormally long, and become complexed together in some places, to form a so-called 'thylakoid plexus', a large irregular network of tubules and perforated thylakoids. A similar set of observations was made by Kirk and Juniper (1967).

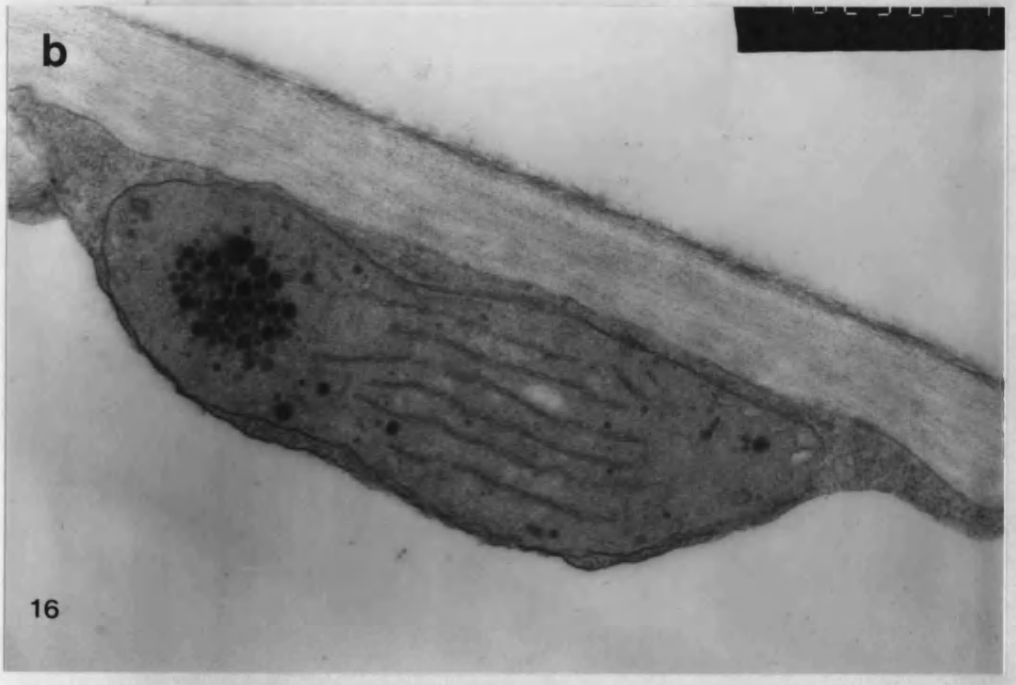
The Lito unripe fruit plastid is very different from the normal Bellboy chloroplast. There are no granal stacks, or large starch grains; instead there is an apparently continuous double membraned or tubular structure, coiled around in the cell. This structure appears to have been cross-sectioned in Figure 2a. This structure is also seen in Orange Bedder fruit (Simpson *et al.*, 1977). In addition, there are a few scattered plasto-globuli, evidence of vesicle formation at the plastid envelope and occasional electron transparent granules, which may be

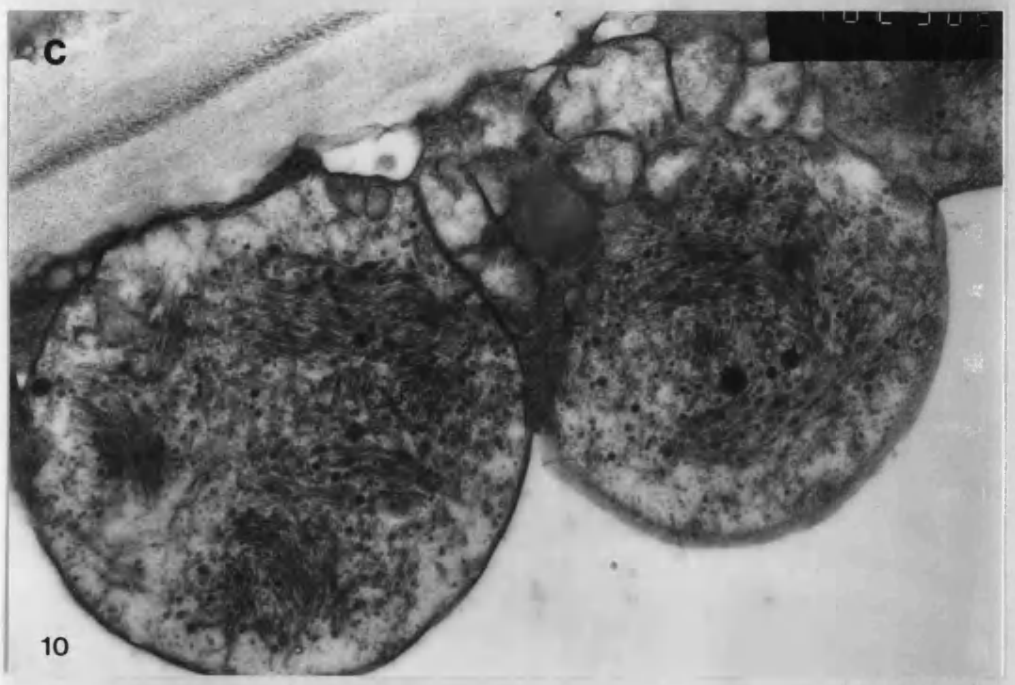
starch granules. The Lito unripe fruit does have some chlorophyll (it has a pale green extract). In the Lito intermediate fruit plastid there is a large cluster of electron-dense plastoglobuli. These are believed to contain the newly synthesized carotenoids. Some vesicles contain very electron transparent structures which are possibly starch grains or carotenoid crystals. The Lito ripe fruit plastid has structure atypical of a normal red fruit plastid. There are no clusters of large electron dense plastoglobuli, although there is the occasional large globule. The plastoglobuli appear small and cluster next to striations believed to be fibrils. The fibrils are thought to give rise to the globules or vice versa (Kirk and Juniper, 1967). The plastid envelope is only vaguely defined, and poorly delineated areas of electron transparency are evident.

Golden Star unripe (green) fruit plastids appear similar to normal Bellboy chloroplasts. There are clearly defined granal stacks and intergranal regions, with a surprising lack of starch granules, although electron transparent areas are visible. There is the occasional plastoglobule, situated near the membranous structures. The intermediate plastid shows drastic loss of this organization. It has developed clusters of plastoglobuli, fibrils, electron transparent granules or vesicles, and in Figure 2e, two fibrillar electron-dense structures can be seen, which have not been pointed out in previous work. The ripe Golden Star (yellow) fruit plastid has

Figure 2. Ultrastructural changes in the plastids of ripening fruit.






c
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d
10

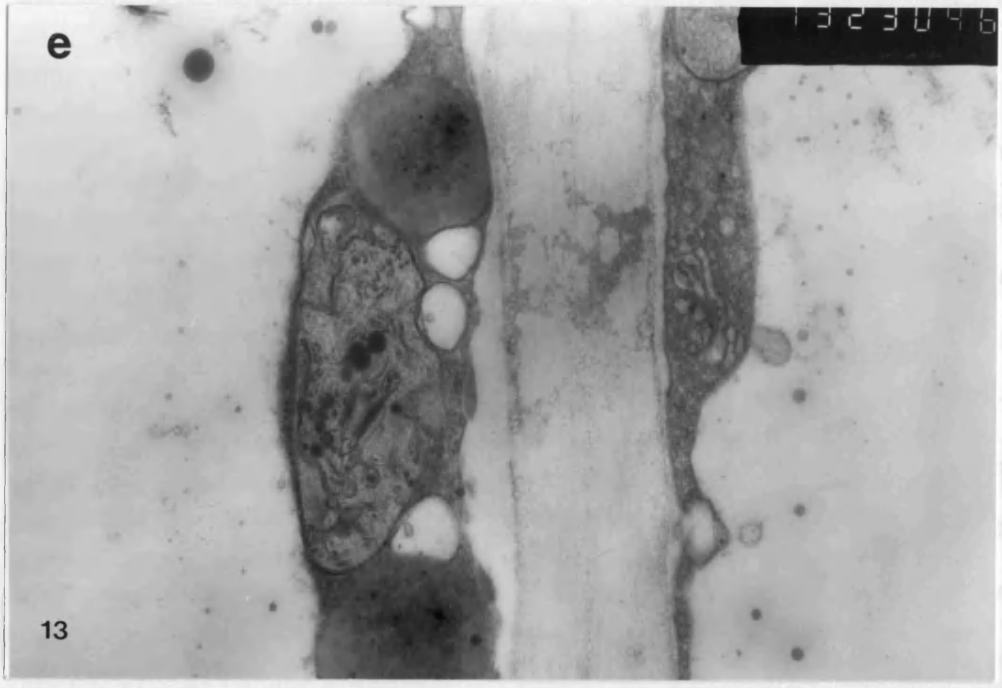


Figure 2. Parts a, b and c show plastids from unripe, intermediate and ripe fruit pericarp tissue of *C.annuum* var. Lito. Parts d, e and f show plastids from unripe, intermediate and ripe fruit pericarp tissue of *C.annuum* var. Golden Star. Fruit tissue was prepared as described in Materials and methods, section 2.2.05. Tissue was sectioned and viewed in a JEOL cx 100 transmission electron microscope. The magnification was between 10 000 and 16 000 and is shown on each figure x 10^{-3}.

occasional plastoglobules, vesicles of an average electron density and a membranous stack, possibly a 'thylakoid plexus'. Dissolution of the middle lamella, seen as an electron dense line in the centre of the cell wall, does not seem to be important during the initial ripening of pepper fruit (Figure 2a-f) in contrast to the case of tomato (Crookes and Grierson, 1983). Very over-ripe fruit do become soft which may be due to dissolution of the middle lamella, or to lack of turgor, but the ultrastructure of fruit at this stage was not studied.

For Golden Star and Lito pepper, fruit ripening consists of a series of ultrastructural changes as in Bellboy fruit. There are differences from Bellboy in the mutant varieties which provide an indication of a lack, or blocking of, particular gene expression. In Lito, lack of normal photosynthetic apparatus means it is not necessary to break it down, with unknown consequences. It is possible that chorophyll breakdown contributes in some way to carotenoid synthesis. The ripe Lito fruit lacks the large clusters of plastoglobuli formed in Bellboy, although to the eye, the fruit are indistinguishable., This raises the question of the function of plastoglobuli, usually considered that of containing the carotenoids. Golden Star fruit are obviously yellow instead of red, and lack the final steps in the biochemical pathway necessary to rearrange antheraxanthin and violaxanthin to produce capsanthin and capsorubin, the red pigments (Davies *et al.*, 1970) (Figure 3, section 1.105). The ultrastructure of the plastid is also

abnormal in that the plastoglobuli and fibrils are much reduced, in number and size. There is a membrane stack shown in the chromoplast in Figure 2F, which is of unknown function. It is possible that lack of ability to produce the red pigment has affected (directly or indirectly) the plastid ultrastructure, or vice versa.

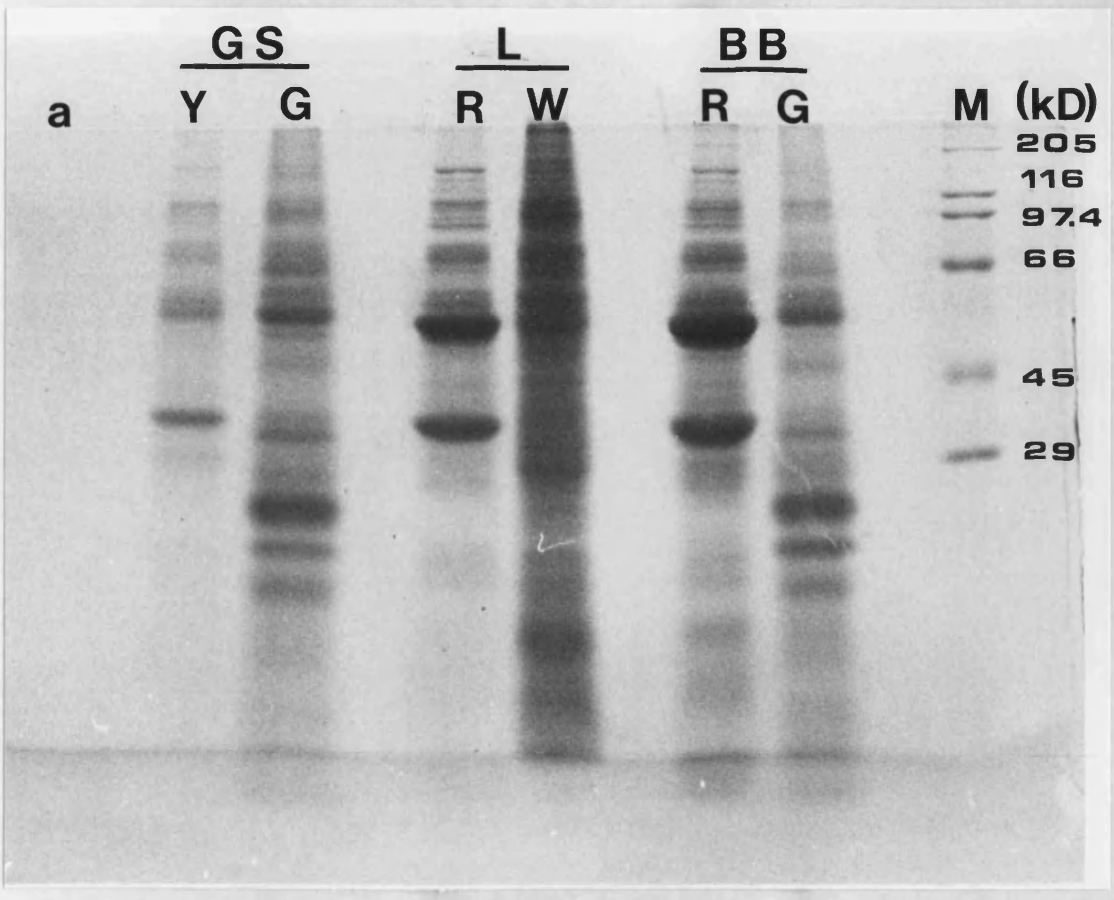
Work linking pigment composition with plastid ultrastructure has been performed by Kirk and Juniper (1967) and Simpson *et al.* (1974, 1977). Kirk and Juniper showed that yellow ripening mutants lacked fibrils, and had some very large osmiophilic globules, which were clumped together. The membranes present in the ripe fruit consisted of long tubules or double membraned sheets. Simpson *et al.* (1974) treated *Capsicum* fruit with 2-(4-chlorophenylthio)ethyldiethylammonium chloride (CPTA) which was shown to induce carotenoid synthesis, and subsequent changes in ultrastructure. This seemed to show that carotenoid synthesis and ultrastructure are related in *Capsicum*. Fibril formation however seems to be under independent genetic control. Simpson *et al.* (1977) worked with different cultivars of orange fruit and showed that fruit that were apparently the same colour had different ultrastructures, indicating that fibril formation was controlled separately from carotenoid synthesis.

2.3.03 SDS-PAGE of plastid proteins.

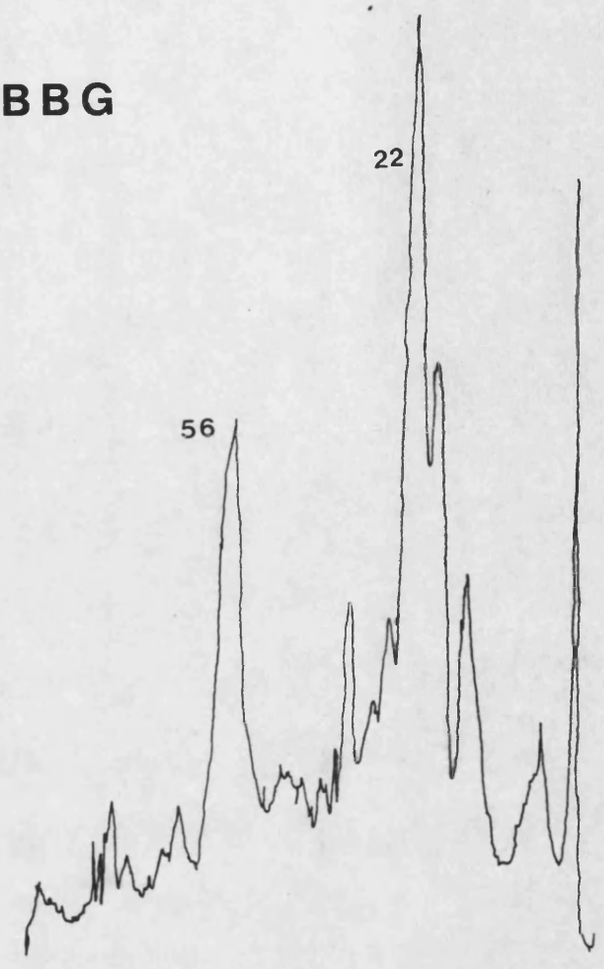
The aim of the protein population study was to establish whether variation of the protein complement took place during ripening, whether new products were present in chromoplasts that were not present in chloroplasts, and to determine the apparent molecular weights of any such products. The information obtained was to be used to facilitate analysis of 2D-gel electrophoresis *in vitro* translation product results, and finally, help in identification of possible ripening-specific cDNAs.

Purified plastids [isolated according to Conover (1986) and Bathgate *et al.* (1985)] were lysed with SDS and stored at a temperature of -20°C to facilitate break up of membranes. The plastid preparation was thawed and boiled in SDS and β-mercaptoethanol for 3 min to inactivate proteases. Difficulty was originally encountered with streaking and poor resolution on gels. This was overcome by centrifuging the boiled sample to pellet insoluble matter. The quantity of sample to be loaded into the wells was determined empirically, by reducing the volume of Buffer B used to resuspend the plastid pellet (section 2.2.06) until a reasonable range of bands was obtained (a typical gel is shown in Figure 3a, scans of each track in Figure 3b-d). The mass of original tissue and final resuspension volume was the same for each fruit type, so that increases or decreases of band quantities represented the differences between the fruit. SDS-PAGE of total fruit protein resulted in a smear, the individual bands of which could not be distinguished.

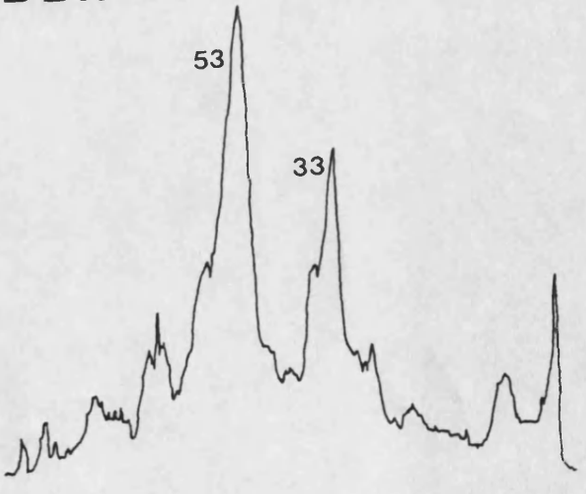
Figure 3. SDS-PAGE of plastid proteins.

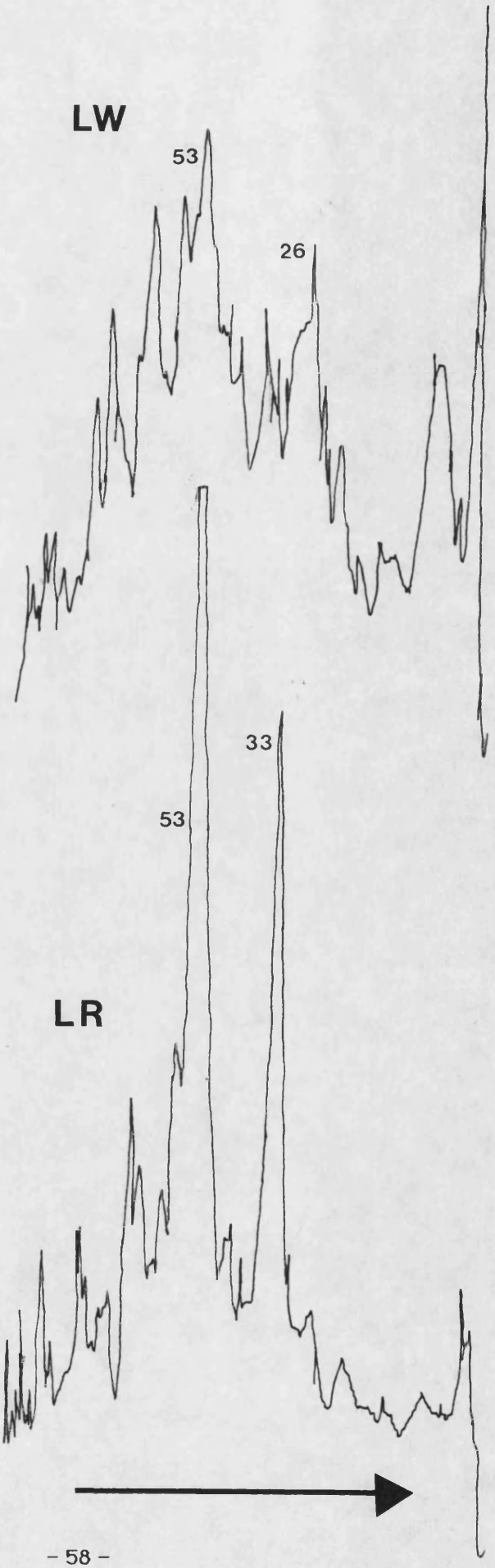


b BBG

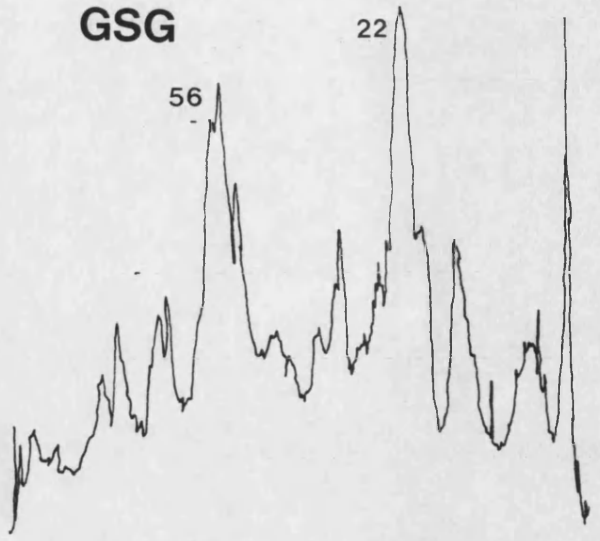


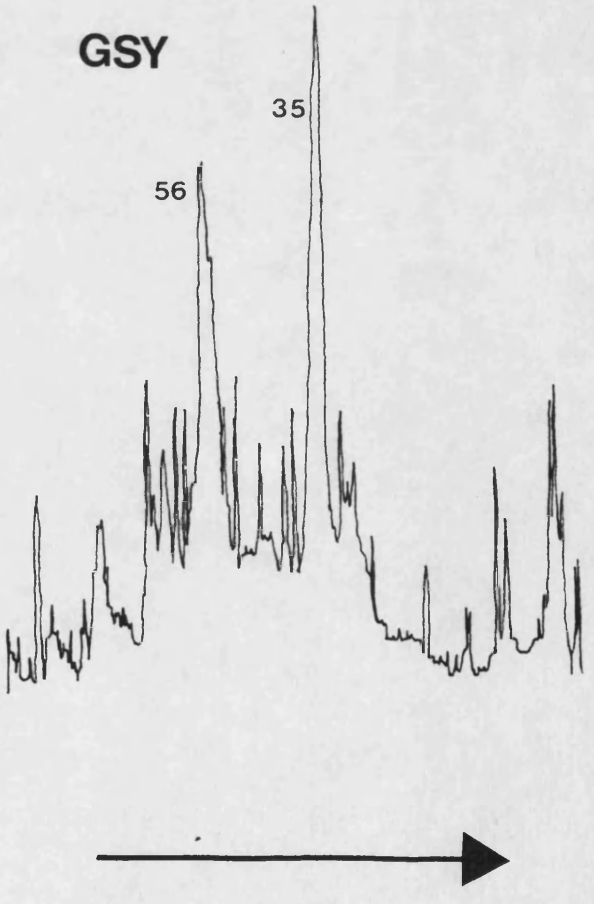
BBR




C
LW
53
26
33
53
LR

d





E

Mol. wts (kD) of polypeptides determined by SDS-PAGE of purified pepper plastids.

Bellboy		Lito		Golden Star	
Unripe	Ripe	Unripe	Ripe	Unripe	Ripe
		205			
		190			
160	160	160	160	160	160
	148	148	148		148
	116		116		116
105	105	105	105	105	105
	99		99		
93	93	93	93	93	93
	87		87		87
	75				
79					
71	71	71	71	71	71
66	66			66	66
		64	64		
61	61		61	61	
		59			
	58		58		58*
56*				56*	56
	53*	53*	53*		
52				52	
	47				
46				46	
	42	42	42		
	49				
36					
				35	35*
	33*		33*		
32				32	
29					
	27		27		
26		26*		26	
	25		25		
22*				22*	
16				16	
	14	14	14		
11	11	11	11	11	
		5			

Figure 3. Part a is a photograph of a typical gel obtained by running plastid proteins in a dissociating system. Samples were prepared by boiling for 3 min in Sample Buffer, followed by a 3 min centrifugation at 12 000 *g* to remove insoluble matter and loaded directly into the gel wells. 10% SDS-polyacrylamide gels were prepared as described in Materials and methods, and run for about 6 hours at 50mA prior to staining and fixing in Coomassie blue solution. Markers are shown in kD. Parts b to d are the traces obtained by scanning the gel tracks in a Joyce Loebl densitometer. The peaks represent bands on the gel and demonstrate differences in the protein populations of unripe and ripe fruit of Bellboy (BBG and BBR), Lito (LW and LR) and Golden Star (GSG and GSY). The direction of electrophoresis is indicated by an arrow. Part e is a table summarizing the results of this protein population survey. The table is so arranged that products common to both ripe and unripe fruit are on the same line; major products are marked with an asterisk (*).

The results presented in Figure 3e can be summarized using Grierson's classification of protein types in ripening fruit (Grierson *et al.*, 1985). He divides the protein population into three classes. (i) Those present only, or in greatly increased amounts in unripe fruit. (ii) Those present in both ripe and unripe fruit. (iii) Those present only, or in greatly increased amounts in ripe fruit. For *C.annuum* var. Bellboy plastids the molecular weights of polypeptides in the three classes are as follows, major products are marked with an asterisk.

(i) 79, 56*, 52, 46, 36, 32, 29, 26, 22* and 16 kD.
(ii) 160, 105, 93, 71, 66, 61 and 11 kD.
(iii) 148, 116, 99, 87, 75, 58, 53*, 49, 47, 42, 33*, 27, 25 and 14 kD.

For *C.annuum* var. Lito plastids the m wts of polypeptides in the three classes are as follows.

(i) 205, 190, 59, 26* and 5 kD.
(ii) 160, 148, 105, 93, 71, 64, 53*, 42, 14 and 11 kD.
(iii) 116, 99, 87, 61, 58, 33*, 27 and 25 kD.

For *C.annuum* var. Golden Star plastids.

(i) 61, 56*, 52, 46, 35, 32, 26, 22*, 16 and 11 kD.
(ii) 160, 105, 93, 71, 66, 32, 26, 22*, 16 and 11 kD.
(iii) 148, 116, 87, 58* and 35* kD.

Pepper chloroplast and chromoplast polypeptides have also been analysed by *in organello* labelling (Powell and Pryke, 1987). Two other fruits have been analysed by SDS-PAGE, and these are the tomato (pericarp tissue, Handa *et al.*, 1983; plastids, Bathgate *et al.*, 1985; Piechulla *et al.*, 1986; Wrench *et al.*, 1987) and the flesh of the avocado (Christoffersen *et al.*, 1982, 1984). It was hoped that a general 'ripening protein' would be found to be at work in pepper and tomato chromoplasts. Tomato is in the same family as pepper (Solanaceae) so there was a chance of finding such a protein in these two fruit. There is a cluster of 'ripening polypeptides' between 32 and 33 kD in tomato chromoplasts (32.5; Bathgate *et al.*, 1985) avocado pericarp (32; Christoffersen *et al.*, 1984) and pepper chromoplasts (33; Powell and Pryke, 1987) which approximately coincide with a ripening specific polypeptide of apparent m wt 33 kD, present in ripe Bellboy and Lito plastids, and 35 kD in Golden Star. The other main ripening product of Bellboy and Lito red fruit has an apparent m wt of 53 kD. This coincides with ripening products of tomato (pericarp, 52 kD; Handa *et al.*, 1983: chromoplast, 53 kD; Bathgate *et al.*, 1985) and avocado pericarp (53 kD, cellulase; Christoffersen *et al.*, 1984). The nearest ripe pepper fruit plastid specific product identified by Powell and Pryke (1987) has a m wt of 48 kD. Comparison of the SDS-PAGE gel scans of pepper chloroplast and chromoplast polypeptides in this paper, with the gel scans reported here, reveals a similar pattern of peaks which means that the Powell and Pryke (1987) ripe fruit product of 48 kD (first major peak) is

probably the same polypeptide as my product of approximately 53 kD (first major peak). The m wts presented in Figure 3E were calculated from the gel rather than from the scans (Figure 3B-D) because it was very difficult to marry the fruit plastid scans correctly with the marker scans. The densitometer scanned from the same zero for each track, but due to the irregular nature of polyacrylamide gels, the actual zero for each track varied slightly. More meaningful and reproducible results were obtained by using the gels to calculate the apparent m wts, and the scans to demonstrate diagramatically the pattern variation of polypeptides between ripe and unripe fruit. This may account for the difference between my results and those obtained by Powell and Pryke (1987), where the gel scans were used to calculate the apparent m wts of the proteins. Lito and Golden Star fruit have a ripening specific product of 87 kD, also reported in tomato chromoplasts (88 kD; Wrench *et al.*, 1987).

The major (Bellboy and Golden Star) green fruit product of 56 kD is presumed to be the large sub-unit (l.s.u.) of ribulose biphosphate carboxylase/oxygenase (rubisco), which has a reported m wt of 53 kD (Grierson and Covey, 1984). The large s.u. of rubisco has also been reported as having a m wt of 50 kD (tomato, Bathgate *et al.*, 1985; pepper, Powell and Pryke, 1987). The m wt of rubisco appears to be over-estimated in this work. This is a consistent finding, however, with the green fruit major peak calculated as between 54 and 56 kD from different gels. The leaf plastid major peak was found to be at

56 kD, again an apparent over-estimate of the 53 kD large s.u. of rubisco. A possible reason for the over-estimate is relative over-loading of that major band, which may make m wt determination imprecise. The small sub-unit (s.s.u.) of rubisco has a reported m wt of 14 kD (Grierson and Covey, 1984) and a corresponding band appears at 15 kD on leaf plastid SDS-PAGE analysis, but is absent from fruit chloroplast preparations. The presence of the large, but not the small s.u. of rubisco seems rather strange, but it is possibly to do with contitutive expression of the large s.u. from the plastome, and inhibition of expression or synthesis of the small s.u. from the nucleus, in fruit. A major peak at the lower end of the m wt scale is at 22 kD for green fruit plastids from Bellboy and Golden Star. This correlates with the results reported by Powell and Pryke (1987) who found major peaks at 21 and 24 kD. Green tomato fruit plastids have a band at 13.5 kD which is assumed to be the small s.u. of rubisco. The unripe Lito fruit displays undetectable levels of the rubisco peaks reported here; its nearest bands in terms of m wt are at 53* kD and 26* kD.

It can be seen from the SDS-PAGE results that Bellboy and Golden Star unripe fruit plastids have similar protein profiles. The green Golden Star fruit appears to lack certain polypeptides present in Bellboy (of m wt 79, 36 and 29 kD), and has one product not detectable in Bellboy of 35 kD. Lito unripe fruit shares approximately 46% of its products with Bellboy green fruit plastids, but otherwise has many unique proteins and has two products (not found in Bellboy or Golden

Star chloroplasts) in common with ripe Bellboy chromoplasts. One of these products is represented as a major peak in ripe fruit plastids (the 53 kD product). Reduced photosynthesis in Lito unripe fruit (indicated by its pale colour) may have resulted in an apparent increase in the relative amounts of proteins normally associated with ripening fruit. The ultrastructure of Lito chloroplasts resembles chromoplasts in the lack of granal structure, and the presence of plastoglobuli.

Golden Star chromoplasts show some products in common with chromoplasts from Bellboy and Lito, but have a general lack of products, particularly in the lower m wt range. A major peak is presented at 58* kD which is represented as only a minor peak in Bellboy and Golden Star chromoplasts. Ripe Golden Star fruit lack the ability to convert γ-carotene to the red pigments, capsorubin and capsanthin, by the necessary cyclization and rearrangement reactions usually associated with chromoplast membranes (Camara *et al.*, 1982). It is possible that a product(s) found in Bellboy and Lito, but not in Golden Star, may represent an enzyme polypeptide associated with these reactions. Except for the Golden Star specific product of 35 kD, found in both ripe and unripe fruits, the remaining ripe Golden Star bands are also found in Bellboy and Lito chromoplasts. The 'rubisco' peak of 56 kD is represented in Golden Star chromoplasts. The possible lingering of some photosynthetic structure indicated by the presence of this product may be reflected in the ultrastructure of the Golden Star chromoplast. Transformation to the normal ripe fruit

chromoplast structure is not as pronounced as in Bellboy fruit, and there is retention of a membranous stack reminiscent of grana. It is possible that there is incomplete disintegration of the photosynthetic apparatus in these fruits.

To summarize: Bellboy and Golden Star chloroplasts show some similarity to the protein profiles of leaves (Powell and Pryke, 1987), with a major peak at 56 kD, thought to be the large sub-unit of rubisco. The mutant white Lito fruit shares some products with ripe fruit, which are not found in unripe fruit of Bellboy and Golden Star. The ripening mutant, Golden Star, with its golden yellow fruit, shows some products normally associated with unripe fruit plastids, and lacks the major ripening specific products of red chromoplasts. The differences in protein profiles between the normal and mutant fruits may reflect differences in gene expression, which may account for the appearance of the mutant fruit.

SDS-PAGE is subject to various limitations which mean that apparent m wts determined in this way may not be accurate. Factors affecting electro-phoretic mobility include abnormal SDS binding, protein conformation, large differences in intrinsic protein charge, and the presence of large side chains (as in glycoproteins). Also several polypeptides may resolve at the same m wt giving the appearance of a single band. Any of these factors may be operating on the pepper fruit plastid ripening associated polypeptides, which means the m wt measurements should be treated with caution. Sharper resolution

may have been obtained by running the gels at 4°C (Piccioni *et al.*, 1982) or using much less sample and a more sensitive staining method such as that of Morrisey (1981). The advantage of using an insensitive method such as Coomassie blue staining is that only the major products are visible. From the point of view of the project, it was only the major products that interested me. In retrospect, investigation of the comparative protein populations of chloroplasts and chromoplasts deserved a more detailed investigation. This work demonstrates differences between fully developed mature green fruit and fully ripened fruit only, intermediate stages were not looked at except in the ultrastructural study. Tomato ripening was studied by Grierson *et al.* (1985) at several stages, and revealed classes of product that could not be revealed in this project.

2.3.04 IEF of plastid proteins.

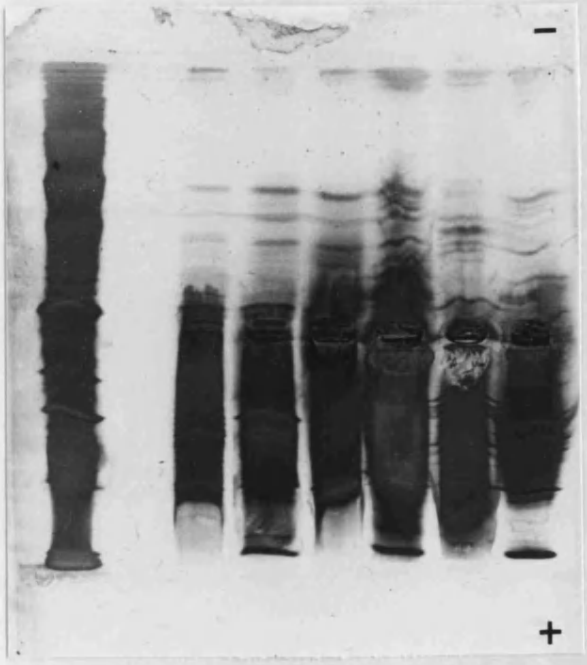
Proteins are amphoteric molecules, bearing amino and carboxyl groups, as well as charged side chains. The overall charge on the protein, which depends on its composition, governs its movement in an electric field along a pH gradient. If the protein is negatively charged at its point of loading it will move towards the gel anode, and if positively charged, it will move towards the cathode. The pI or isoelectric point of a protein is defined as the pH at which the anionic and cationic values are in balance and the protein has no net charge. Hence its migration ceases and it is said to 'focus' at that point.

Isoelectric focusing is a technique which involves the separation of proteins in a pH gradient with the aim of identifying proteins by their isoelectric points (Isoelectric Focusing. Pharmacia.) Initial attempts at isoelectric focusing involved the use of rod gels. The gels were found to be very difficult to remove from the tubes, for staining, and also expensive in terms of the amount of ampholyte required. The Pharmacia PhastGel system was used because of its simplicity. The other advantage of this system was that all the protein samples are run under the same conditions which is useful for comparative purposes. A drawback of the system is the minute size of the gels, which limits the volume of sample that it is possible to load. A pH gradient is created across about 3.5 cm, and the numerous proteins present within a crude sample, such as plastid proteins, are focused within that space. It made the measurement of distances difficult, and great care had to be taken to maintain a relevant zero from which the distances were measured.

Most plastid proteins have pIs between pH 3.5 and 6.0. By heavily overloading the gel it is possible to see proteins with alkaline pIs but these are present in low amounts compared with the proteins with more acidic pIs (Figure 4A,B). Approximately the same number of proteins were identified with this method as using SDS-PAGE. The proteins can be similarly divided into classes. Class (i) those proteins present in only or in greatly increased amounts in unripe fruit; class (ii) proteins present in both ripe and unripe fruit; and class (iii)

Figure 4. IEF of plastid proteins.

a



b

pIs of proteins determined by iso-electric focusing of purified pepper plastids.

Bellboy		Lito		Golden Star	
Unripe	Ripe	Unripe	Ripe	Unripe	Ripe
3.60*	3.60			3.60*	
	3.70				
3.75*	3.75*	3.75*	3.75*	3.75*	3.75*
3.95	3.95			3.95	
					4.05
				4.15	4.15
		4.20			
4.30	4.30			4.30	
4.40*	4.40*	4.40		4.40*	4.40
			4.50*		
4.60*		4.60	4.60	4.60*	4.60*
		4.75	4.75		
4.80*	4.80*		4.80	4.80*	
	4.90		4.90		4.90
					5.15
					5.30
					5.55
				6.55	6.55
6.70	6.70			6.70	6.70
				6.75	
6.80		6.80	6.80		
6.90		6.90		6.90	6.90
	7.10	7.10	7.10	7.10	7.10
7.25	7.25	7.25		7.25	7.25
		7.50			
7.75	7.75	7.75	7.75	7.75	7.75
8.00	8.00	8.00	8.00	8.00	8.00
8.20	8.20	8.20	8.20	8.20	8.20
>9.30	>9.30	>9.30	>9.30	>9.30	>9.30

Figure 4. Samples were prepared by lysing purified plastids with a non-ionic detergent, Triton X-100. The samples were lysed, frozen-thawed twice and loaded directly onto the pre-run ready-prepared Phastgel gel as described in Materials and methods, section 2.2.08(i). The gel was run for 425 volthours against a commercial mixture of proteins of known pIs. The gel was silver-stained automatically in the staining chamber of the Phastgel apparatus. Details of the staining programme are given in Materials and methods, section 2.2.08(ii). Part a is a photograph of a typical gel, with pI markers shown to the left and the lanes left of the markers are as follows: Golden Star, yellow; GS, green; Lito, red; Lito, white; Bellboy, red; BB, green. Part b is a table summarizing the results of this protein population survey. Major products are marked with an asterisk (*). (+) and (-) indicate the anode (acidic polypeptides) and cathode (alkaline polypeptides), respectively.

proteins present only or in greatly increased amounts in ripe fruit. For *C.annuum* var. Bellboy plastids, the pIs of the three classes of protein are as follows:

(i) 4.60*, 6.80 and 6.90.
(ii) 3.60, 3.75*, 3.95, 4.30, 4.40*, 4.80*, 6.70, 7.25, 7.75, 8.00, 8.20 and >9.30.
(iii) 3.70, 4.90 and 7.10.

For *C.annuum* var. Lito plastids:

(i) 4.20, 4.40, 6.90, 7.25 and 7.50.
(ii) 3.75*, 4.60, 4.75, 6.80, 7.10, 7.75, 8.00, 8.20 and >9.30.
(iii) 4.50*, 4.80 and 4.90.

For *C.annuum* var. Golden Star plastids:

(i) 3.60, 3.95, 4.30, 4.80* and 6.75.
(ii) 3.75*, 4.15, 4.40*, 4.60*, 6.55, 6.70, 6.90, 7.10, 7.25, 7.75, 8.00, 8.20 and >9.30.
(iii) 4.05, 4.90, 5.15, 5.30 and 5.55.

The method is limited in that only proteins with pIs between 3 and 10 will be focussed, and only proteins visible with the staining method used will be seen. Some proteins have been shown to stain with Coomassie blue, but not silver nitrate (Morrisey, 1981). Proteins may interact and aggregate, causing co-migration, and the pI thus calculated is that of the

aggregate rather than the individual proteins. Centrifugation prior to electrophoresis was performed to prevent streaking on the gels, and this may have removed aggregates and also some genuine proteins.

IEF has been used frequently to separate mixtures of plant proteins (maize, Hagan and Rubenstein, 1980; pea, de Vries *et al.*, 1982; oat, Fabijanski *et al.*, 1985), usually in conjunction with SDS-PAGE to separate those proteins with known pIs according to m wt. There are few reports in the literature of ripening proteins being separated by IEF. One of the aims of becoming familiar with the IEF technique was eventually to perform 2D gel electrophoresis of plastid proteins and identify proteins by both m wt and pI. These experiments are described in the next section (section 2.3.05).

2.3.05 2-Dimensional gel electrophoresis of purified plastid proteins.

The aim of the 2D gel electrophoresis was to study more specifically the variation in protein population between ripe and unripe fruit plastids. Groups of polypeptides of similar m wt or pI, which migrated together on SDS-PAGE or IEF respectively, could be separated according to their dissimilar property in the other dimension.

Purified plastid proteins were separated by IEF in a pH gradient of 3-10. The method used was essentially that of IEF

described in section 2.2.08. The lane in which the proteins were separated was identified by the central indentation caused by the comb, and the strip cut out from the gel. The anode and cathode were marked and the gel equilibrated, to remove the ampholytes and to saturate with SDS-PAGE running buffer. The strip was then transferred to the top end (cathode) of a PhastGel, acrylamide gradient 10-15%, and the proteins run according to the SDS-PAGE method described in section 2.2.07, against a set of marker proteins. The 2D gels were silver stained as described in section 2.2.09(iv).

The spots obtained (see Figure 5A for examples of Bellboy ripe and unripe plastid protein profiles) were analysed according to pI and m wt and the results summarized in Figure 5B. Analysis was performed by mounting the gels on millimetre graph paper and comparing the positions of the spots with those of the markers. As in Grierson *et al.* (1985) the products could be divided into three classes. (i) Those present only or in greatly increased amounts in unripe fruit plastids. (ii) Those present in both ripe and unripe fruit plastids. (iii) Those present only or in greatly increased amounts in ripe fruit plastids. For *C.annuum* var. Bellboy plastids the classes contain the following, given as (pI, m wt in kD);

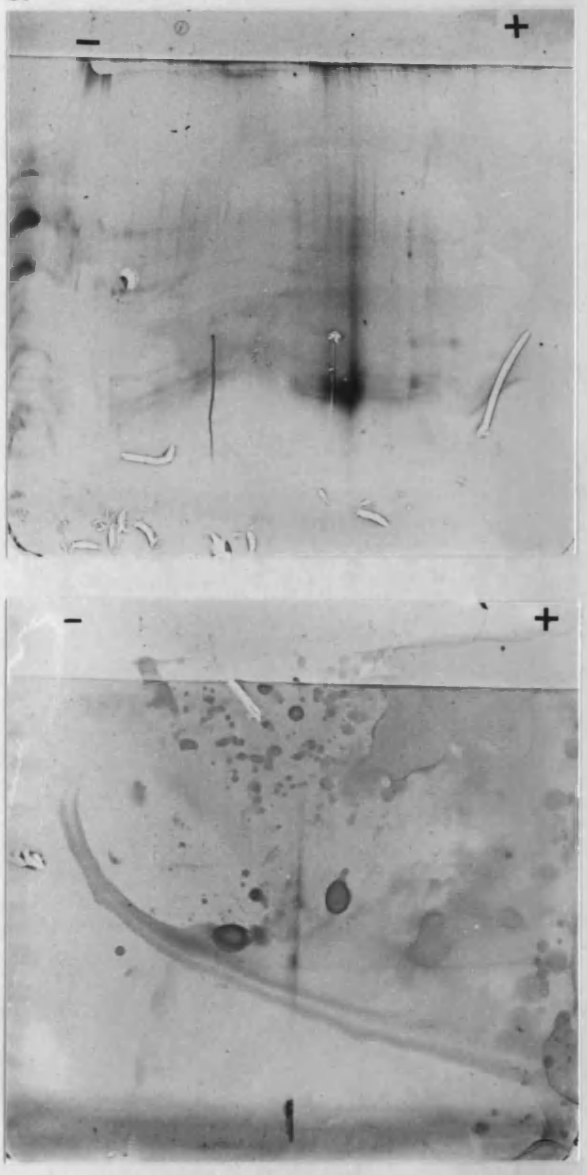
(i) 4.80,72; 4.80,62; 4.80,47; 4.80,41; 4.80,31; 4.80,27; 4.80,23.5; 4.30,31; 4.30,23.5.

(ii) 4.80,33.5; 4.80,25.5; 4.80,22; 4.80,20.5.

(iii) 4.80,42; 4.80,12.5.

Figure 5. 2D gels of purified plastid proteins.

a



b

2D-gel electrophoresis of plastid proteins (pI, m wt in kD).

Bellboy		Lito		Golden Star	
unripe	ripe	unripe	ripe	unripe	ripe
					4.95, 41
					4.90,125
					4.90,105
					4.90, 88
			4.80,83		
					4.90,77
4.80,72			4.80,72		
					4.90,66
4.80,62					
				4.80,58	4.90,58
			4.80,54		
				4.80,51	
					4.90,50.5
			4.80,47.5		
4.80,47					
					4.90,44
	4,80,42				
4.80,41					4.90,41
					4.90,37.5
					4.90,36
4.80,33.5	4.80,33.5	4.75,33.5			
4.80,31					
4,80,27					4.90,27
4.80,25.5	4.80,25.5				4.90,25.5
4.80,23.5					
4.80,22	4.80,22				
4.80,20.5	4.80,20.5				4.90,20.5
		4,75,19.5			
					4.90,16.5
	4.80,12.5				
				4.80,12	
				4.30,62	
				4.30,48	
4.30,31					
4.30,23.5					
				4.30,20.5	
					4.15,82
					4.15,62
4.15,23.5					
					4.15,18
				4.15,13.5	
				4.15,12.5	
		3.95,83			
3.95,31					
				3.95,20.5	

2D-gel electrophoresis of plastid proteins (pI, m wt in kD).

Bellboy		Lito		Golden Star	
unripe	ripe	unripe	ripe	unripe	ripe
					3.90,62

Figure 5. Plastid proteins were treated with 2D Sample Buffer as described in Materials and methods, section 2.2.09(i), and applied to the first dimension. This consisted of an IEF gel, similar to that described in the legend to Figure 4. Gel tracks were cut from the gel after running and equilibrated as described in section 2.2.09(ii), prior to staining with bromophenol blue and applying to the second dimension, which consisted of an SDS-PAGE gel (10 to 15% gradient). This gel was run until the dye reached the gel foot. The gel was silver-stained similarly to the IEF gels of Figure 4. Details of the staining procedure are given in Materials and methods, section 2.2.09. Part a shows the gel obtained from Bellboy unripe green and Bellboy ripe red fruit, part b is a table summarizing the results of this protein population survey. Values are represented as pI, and m wt in kD.

For *C.annuum* var. Lito:

(i) 4.75,33.5; 4.75,19.5; 3.95,83.

(ii) no products.

(iii) 4.80,83; 4.80,72; 4.80,54; 4.80,47.5.

For *C.annuum* var. Golden Star:

(i) 4.80,51; 4.80,12; 4.30,62; 4.30,48; 4.30,20.5; 4.25,13.5; 4.00,20.5.

(ii) 4.90,58.

(iii) 4.95,41; 4.90,125; 4.90,105; 4.90,88; 4.90,77; 4.90,66; 4.90,50.5; 4.90,44; 4.90,41; 4.90,37.5; 4.90,36; 4.90,27; 4.90,25.5; 4.90,20.5; 4.90,16.5; 4.20,82; 4.20,62; 4.20,18; 3.90,62.

The ranges of m wts obtained (Bellboy, 72-12.5 kD; Lito, 83-19.5 kD; Golden Star, 125-16.5 kD) are smaller than those obtained by SDS-PAGE implying loss of some products. Regarding specific m wts, approximately 40% overall of 2D m wts fall within 1 kD of an SDS-PAGE determined value, the others do not. Of 'ripening specific' products, those identified by SDS-PAGE coincide with the m wts of spots on the 2D gels only in the following cases (the m wts given are those determined by SDS-PAGE);

Bellboy (ripe fruit specific), 42 kD.

Lito (ripe fruit specific), no products.

Golden Star (ripe fruit specific),35 and 88 kD.

The ranges of pI of product obtained by 2D gel electrophoresis are much smaller than those obtained by the IEF technique, again implying loss of some products. In 2D analysis only acidic pIs are represented, with a concentration at approximately pI 4.85 in each type of fruit. The pI of 4.85, which represents the majority of 2D products in all the fruit, is not represented specifically in the IEF data although pIs of 4.80, 4.75, and 4.90 are. As there was some ambiguity regarding the precise pIs of the 2D products, the pIs were keyed to those values (already defined in section 2.3.04) which were nearest to the approximate value determined from the 2D gels. For Bellboy green and red fruit, Lito red, and Golden Star green this meant a shift of -0.05 pH units, from 4.85 to 4.80; for Lito white fruit, a shift of -0.10 pH units from 4.85 to 4.75; and for Golden Star yellow fruit, a shift of +0.05 pH units from 4.85 to 4.90. Of 'ripening specific' products those identified by 2D gel electrophoresis, which coincide approximately with defined IEF values are as follows:

Bellboy (ripe fruit specific), pI 4.80 (m wts 42, 12.5 kD).
Lito (ripe fruit specific), pI 4.80 (m wts 83, 72, 54, 47.5 kD)
Golden Star (ripe fruit specific), pI 4.95 (m wt 41 kD), pI 4.90 (m wts 125, 105, 88, 77, 66, 50.5, 44, 41, 37.5, 36, 27, 25.5, 20.5, 16.5 kD), pI 4.15 (m wts 82, 62, 18 kD).

The possible m wts of the products are indicated. From these analyses a tentative identification of a chromoplast specific polypeptide of *C.annuum* var. Bellboy may be made at pI 4.80, m wt 42 kD; and for *C.annuum* var. Golden Star at pI 4.90, m wts 87 and 35 kD. No other 2D products are consistent with both the IEF and the SDS-PAGE results and this inconsistency casts doubt on the identification of ripe fruit specific products from Bellboy and Golden Star, which may be coincidental. Major peaks such as Bellboy, and Golden Star chloroplast ('rubisco') 56 kD, and Bellboy and Lito chromoplast 53 and 33 kD are not strongly defined on the 2D gels..

Comparison of the ripe fruit specific products with the literature is difficult due to lack of comparable studies, although Christoffersen *et al.* (1984) performed 2D analysis of *in vitro* translation products of ripe avocado pericarp. The pIs and m wts of ripe fruit associated products in this study were: 6.3,80; 5.1,36; and 5.7,16.5 which do not correlate with any of the products reported in this work. A ripe fruit specific product of a similar m wt to the Bellboy 42 kD product was identified in tomato as polygalacturonase (43 kD, Handa *et al.*, 1983) but this tomato product is likely to be cytoplasmic rather than plastidic, as polygalacturonase acts on the cell wall rather than in the plastid. With regard to the products identified in Golden Star, a major ripe fruit specific product of tomato chromoplasts is described (88 kD; Wrench *et al.*, 1987).

On a gross level the patterns of spots obtained using 2D gel electrophoresis were similar for all types of fruit. The pattern typically consisted of a streak of products at about pI 4.85, sometimes distinguishable into separate spots, sometimes not. On the anodic side of this streak were other spots, much fewer, arranged in various ways. The main problems in interpretation were lack of spots on the gels, ambiguity regarding the marker proteins, lack of correlation with SDS-PAGE and IEF results for each type of fruit, and internal lack of correlation between fruits that logically should display some similarity (e.g. Bellboy red and green fruits). These problems will be dealt with in the following paragraphs.

Lack of spots on the gels, i.e. far fewer products than one would expect from the SDS-PAGE, and IEF results, could have a number of causes. Light loading of the first dimension and subsequent distribution and dilution of signal in the second dimension could result in apparent loss of some spots. These results were produced from overloading an IEF gel as determined necessary to visualize faint alkaline pIs, but this quantity may be insufficient for 2D analysis. Loss of protein could also have occurred during the equilibration steps described in section 2.2.09(ii). At this stage the proteins are not fixed in the gel and incubation in buffer could cause them to diffuse out. Cutting the strip from the first dimension IEF gel is done using the central indentation of the comb to denote the centre of the strip. A straight strip is cut, which assumes straight running of the products. If the lane was curved for

any reason (such as variations in electric field), products would be lost. There is no way of telling where the proteins are on the first dimension gel, except by the slight comb indentation. Single sample tube gel electrophoresis of the first dimension avoids these problems but as previously noted, difficulty was encountered with other aspects of this method. Also the method of staining may not be suitable for visualization of all the products and some may remain unstained and therefore 'lost'.

Marker proteins for the SDS-PAGE dimension were run on each gel, although sometimes the bands ran badly or were barely visible due to edge effects. The markers used for the IEF dimension, however, were run on a separate gel from the products in question, and calculation of the pIs of products on the gels was made using the distribution of marker proteins on these separate gels. That this is ambiguous is demonstrable by observation of the calibration curve for the marker proteins (not shown). Three sets of markers were run on different gels and the results combined to produce a calibration curve. Variation of running distance, in the most extreme cases, accounts for an ambiguity at some points of over 1 pH unit. The curve used to calculate the pIs of products was an average of these three curves, which means that any one pI could be 0.5 pH units in either direction. This renders the specific identification of products by pI a relative, rather than absolute, exercise. Combination of the various problems associated with loss of data and ambiguity of pI markers led

to lack of correlation with the more rigorously controlled SDS-PAGE and IEF data. Reasons for lack of internal correlation within the experiment (for example, between fruit of the same variety) are more difficult to explain, but are probably related to the various ways in which protein may have been lost, and the unreliability of the marker proteins.

To summarize, the technique of 2D gel electrophoresis using the Pharmacia PhastGel system requires extensive optimization before any firm conclusion can be drawn from data so produced. The results presented here represent a preliminary foray into this field of experimentation and provide a useful baseline for further study, but are not a certain means of positive identification of ripe fruit specific polypeptides, by pI and m wt.

The aims of this section of work were largely achieved in that I showed that there was variation of the protein population between unripe and ripe fruit, that ripe fruit plastids contained products not present in unripe fruit plastids and the apparent molecular weights of these products were determined. The changes in protein population were concurrent with the extensive pigment and ultrastructural alterations previously described, and implied that ripening is a dramatic developmental process, involving both destruction and generation, probably controlled at the level of gene expression.

Chapter 3. Extraction and study of pepper fruit RNA.

3.1.00 Introduction.

The aim of the second phase of experiments was to analyse the messenger RNA population of ripening fruits; determining the seat of control (plastidic or nuclear), which particular messages increased or decreased, and whether any correlations could be drawn with the results of the protein analysis. The method of extraction had to be approached from first principles and a method developed which produced RNA of a quality and quantity suitable for the experiments to be performed. RNA extraction procedures necessarily involve disruption of the subject tissue. As the cells are broken up, RNases are released to mix freely with the RNAs. Most information about RNase is based on studies of bovine pancreatic RNase A, which is produced to digest exogenous RNA from the diet, e.g. the RNA of bacteria in the rumen (Boyer, 1971, 1982). Most cells contain RNase, possibly involved in the controlled degradation of RNA. Cytoplasmic RNA is synthesized directly into the cisternal side of the rough endoplasmic reticulum by ribosomes bound to the surface, and therefore, is not normally free in the cytoplasm. It is a very stable enzyme, which becomes denatured upon absorption into solvents such as ethylene glycol, formamide, and phenol, but renatures rapidly in

water to recover full activity. RNase A is solubilized and inactive in high concentrations of SDS at high pH. Methods of RNA extraction involve buffers containing chaotropic agents which disrupt the structure of RNase while the RNA is separated from it. The essence of extraction is speed; eliminating nucleolytic degradation of RNA depends on the rate of denaturation of RNase exceeding the rate of RNA hydrolysis. The initial few seconds of disruption are therefore very important (Chirgwin *et al.*, 1979). Various methods of extraction were tried, and the one chosen was that which gave the most useful results in terms of yield, purity, and efficiency of translation. The final method was a combination of two other methods with an alteration designed to defeat RNases. Once a method was chosen, RNA extraction was followed by poly A+ RNA purification, and *in vitro* translation. In spite of using established methods performing the extraction and *in vitro* translation did not prove to be easy, as will be discussed in the chapter. The aims of the work were largely fulfilled despite the difficulties encountered, and I was able to study, by *in vitro* translation, the changes to the RNA population in ripening pepper fruit. The background to the mRNA studies is discussed in the General Introduction, sections 1.1.07 (nuclear control of ripening) and 1.1.09 (ripening as a developmental process, involving changes in the mRNA population).

3.2.00 Materials and Methods.

3.2.01 Plant material.

Plants were grown as in section 2.2.01, and the fruit chosen on the basis of their colour which was a good guide to their ripeness. Fruit used for RNA work was always handled with gloves to avoid contamination with RNase.

3.2.02 Chemicals.

All the chemicals used for the extraction and treatment of RNA were of the highest possible purity, from BDH, Sigma or Aldrich. The water used was purified through a milliQ filtration system, and also sterilized by autoclaving. All buffers and equipment coming into contact with the RNA were sterilized by autoclaving, dry heat (200°C for 5 hours), or soaking in 0.1% diethyl-pyracarbonate (DEP) in ethanol. Crystalline phenol was redistilled at 160°C before use, as described in Maniatis *et al.* (1982). Oligo (dT) cellulose was obtained from Sigma, amino acid depleted rabbit reticulocyte lysate, ^{35}S-methionine, amino acid mixture (minus L-methionine) and pure potassium and magnesium solutions were obtained from Amersham International.

3.2.03 GuSCN method of RNA extraction. (Guanidine thiocyanate)

This method was that recommended by P and S Biochemicals Ltd. and is an adaption of the method pioneered by Chirgwin et al (1979), itself a progression from the method developed by Harding *et al.* (1977), which uses guanidium hydrochloride.

(i) Extraction.

Fruit pericarp tissue was ground to a fine powder under liquid nitrogen and allowed to thaw in GuSCN solution (4.00M GuSCN; 0.5% sarcosyl; 25mM sodium citrate, pH 7.0; 0.10M β-mercaptoethanol; filtered before use and kept in a dark bottle.). The mixture was warmed to 37°C to ensure thawing, and vortexed 2-5 times in 10 second bursts, prior to centrifugation at top speed in a bench centrifuge to pellet cell debris. The supernatant was made to 1g per ml CsCl, and layered onto a cushion of CsCl solution (5.70M CsCl; 0.10M EDTA, pH 7.0; 0.2% DEP. This solution was autoclaved before use to hydrolyse the DEP which may otherwise inactivate the RNA.) and centrifuged at 130,000*g* (37,000 r.p.m. in a Beckman SW50.1 head) for 22 hours at 15-20°C. This was to pellet the RNA through the CsCl cushion. At the end of the run the pellet was washed in sterile TE buffer (10mM Tris-HCl, pH 8.0; 1mM EDTA) and then resuspended in TE and dissolved over a few hours on ice.

(ii) Ethanol Precipitation.

The RNA was then ethanol precipitated, as described in Maniatis *et al.* (1982). The RNA solution was made to 0.30M salt with ammonium acetate and precipitated under 2.5 volumes of absolute ethanol at -20°C overnight. The RNA was recovered by centrifuging at top speed in a microfuge, at 4°C, for 15 minutes. The pellet was drained, and dried under vacuum, and resuspended in pure water for storage at -80°C.

3.2.04 Phenol extraction and CsCl centrifugation.

This procedure was recommended by Conover (1986), and the initial extraction at least, is very similar to most of those found in the literature.

(i) Extraction.

Phenol (prepared as described in Maniatis *et al.*, 1982) was equilibrated with nucleic acid extraction buffer (0.10M Tris-HCl, pH 9.0; 5mM EDTA; 0.40M NaCl; 0.02% SDS) and chloroform in a ratio of buffer:phenol:chloroform 2:1:1. ('chloroform' refers to a mixture of chloroform : isoamylalcohol, 24:1.) The aqueous phase of this mixture was taken off and the tissue was homogenized in it at a concentration of 1g of tissue per 4ml of buffer, in a glass Waring blender. The homogenate was filtered through sterile muslin into the stirring organic phase, and stirred at 4°C for 15 minutes. The mixture was centrifuged at top speed in a bench centrifuge to separate the phases, and the top

aqueous phase was re-extracted twice more with an equivolume of phenol:chloroform 1:1. The final aqueous phase was extracted with chloroform only, to remove traces of phenol. The nucleic acids were ethanol precipitated as described in section 3.2.03 (ii) and prepared for loading on a CsCl gradient.

(ii) CsCl gradient centrifugation.

This technique was first described by Radloff *et al.* (1967). The sample was resuspended in SSC buffer (0.14M NaCl; 15mM sodium citrate, pH 7.0) and made to 1g per ml CsCl. 50µl of 10mg/ml ethidium bromide was added to each tube volume of 4.5ml. The tubes were heat sealed, and the gradients centrifuged at 50,000 r.p.m. in a Beckman Vti65 rotor, at 15°C, for 12 hours. The gradients were carefully decelerated under slow brake, and viewed in UV light. The RNA could be seen as a strip down the side of the tube and the DNA as a band floating about midway down the tube. The tops were cut off the tubes and the DNA taken off with a hypodermic needle. The RNA was rinsed in sterile TE buffer and then resuspended in sterile TE.

(iii) Removal of ethidium bromide.

Ethidium bromide was removed from the RNA by repeated extraction with butan-1-ol, or isoamylalcohol. This also reduced the volume of the sample, so if the volume decreased

too much, water saturated solvent was used. The RNA was then ethanol precipitated as described in section 3.2.03 (ii).

3.2.05 High SDS method of RNA extraction.

This method was recommended by Dr. J.Beeching of Bath University. Tissue was weighed and ground to a fine powder under liquid nitrogen. The frozen powder was added to JB extraction buffer (50mM Tris-HCl, pH 9.0; 0.15M LiCl; 5mM EDTA; 5% SDS) and the frozen slurry allowed to thaw for about 3 minutes. It was then strained through sterile muslin into an equivolume of phenol:chloroform 1:1. The mixture was stirred for 15 minutes at 4°C, and then centrifuged at top speed in a bench centrifuge to separate the phases for 15 minutes. The aqueous phase was taken off and allowed to extract in a second equivolume of phenol:$CHCl_3$ as the organic phase underwent a back extraction with JB washing buffer (20mM Tris-HCl, pH 9.0; 2mM EDTA). The aqueous phases were combined and extracted twice more with phenol:$CHCl_3$, and once with $CHCl_3$ alone. The final aqueous phase was taken off into a sterile measuring cylinder to estimate its volume and made to 0.2M with solid LiCl. This total nucleic acid preparation was put under 2.5 volumes of ethanol at -20°C overnight to precipitate.

The precipitate was collected by centrifugation at 10,000*g*, 4°C, for 20 minutes and the pellets resuspended in 3.00M

sodium acetate, pH 5.5, and combined. The centrifugation and resuspension were repeated twice more, to dissolve out DNA and polysaccharide. The final pellet was resuspended in water, and left to precipitate overnight at 4°C, under 2 volumes of 3.00M LiCl.The final precipitate was collected by centrifugation as before, and ethanol precipitated as described in section 3.2.03 (ii).

3.2.06 Final method of RNA extraction.

The method finally adopted for the RNA extractions was a combination of the aqueous phenol method, section 3.2.04, and the high SDS method, section 3.2.05. The method is essentially that of section 3.2.05 except that instead of freezing the tissue it was homogenized directly in JB extraction buffer that was saturated with the phenolic phase. The homogenate was filtered through muslin into the stirring phenolic phase, and continued from there, as described in section 3.2.05.

3.2.07 Measurement of yield.

RNA was resuspended in a known volume of water and 10µl of this solution was diluted into 0.5ml of water in a quartz cuvette. The absorbance of the RNA solution at 260nm was compared to a blank of water only. The concentration of RNA in the diluted sample was calculated assuming:

1 A_{260} = a concentration of 40µg/ml.

3.2.08 Measurement of purity.

The purity of an RNA sample was expressed as the ratio of its absorbance at 260nm and that at 280nm, the former being the peak of absorbance for nucleic acids and the latter being the peak of absorbance of protein and phenol. A pure RNA sample should have an Abs 260/Abs 280 ratio of 2 or more.

3.2.09 Scanning samples to demonstrate purity.

RNA was resuspended as described in section 3.2.07, and the solution scanned in a Unicam SP 1800 spectrophotometer, within a suitable range of wavelengths. The relative absorbance at each wavelength was plotted out on a chart recorder.

3.2.10 Tube gel electrophoresis of RNA.

This method was adapted from Conover (1986) and Grierson (1982). 2.4% polyacrylamide gels were made by mixing the following solutions. 1.6ml of 15% acrylamide/0.75% bis-acrylamide, 3.3ml of BE buffer (6mM EDTA; 0.12M Tris-HCl, pH 7.8; 0.01M sodium acetate), 5.1ml of distilled water, 100µl

of freshly made up 10% ammonium persulphate and after de-gassing, 10µl of TEMED. The gel mixture was poured into upright quartz tubes sealed at one end, and left to polymerize for about an hour. The gels were pre-run for 30 minutes in running buffer (2mM EDTA; 0.04M Tris-HCl, pH 7.8; 0.033M sodium acetate; 0.05% SDS) at 4mA per gel.

RNA was dissolved in electrophoresis buffer (0.036M Tris-HCl, pH 7.7; 0.03M NaH_2PO_4; 1mM EDTA; 0.2% SDS; 10% sucrose), such that 30µg of RNA could be loaded onto each gel. Bromophenol blue dye was run in an adjacent tube to observe the movement of the gel front. The gels were run at 4mA per gel for about 2 hours or until the dye had just run off. They were then scanned in a Fisons/Isco tube gel scanner. The relative absorbances of each part of the gel were plotted on a chart recorder.

3.2.11 Denaturing gel electrophoresis of RNA.

The method used is a modification of the technique described by McMaster and Carmichael (1977), and is that recommended by Hatfield Polytechnic in their Nucleic Acids Workshop.

(i) Sample preparation.

RNA was dissolved in 10mM phosphate buffer pH 7.0 at a concentration of 0.25µg/µl. RNA to be denatured was treated with 8µl of GFP mix. If a native sample was to be run

alongside the denatured sample, it was treated with 8µl of 10mM phosphate buffer. GFP mix was made by mixing 6ml of deionized glyoxal with 4ml of deionized formamide, plus 0.1ml of 10mM phosphate buffer pH 7.0, and 3.75ml of sterile distilled water. Glyoxal was deionized by stirring with several changes of Bio-Rad mixed bed resin AG50 -X8 (D), and formamide was deionized by stirring with several changes of BDH Amberlite monobed resin MB3. GFP mix was stored at -80°C in full sterile polypropylene tubes, and the formamide was stored at -20°C in covered bottles. Those samples containing GFP were heated at 55°C for 15 minutes. Prior to loading, 2µl of loading mix was added to each sample (20% w/v sucrose; 10% w/v Ficoll; 10mM EDTA; 1% bromophenol blue; 10mM phosphate buffer pH 7.0).

(ii) Running the gel.

A 1.1% agarose gel in 10mM phosphate was prepared by autoclaving the agarose suspension. The suspension was allowed to cool to 56°C prior to pouring into a sterilized gel mould. Samples were loaded into the wells using a Gilson P20 pipette, and the gel was run at 100v with rapid circulation of buffer for 1 hour.

(iii) Staining the gel.

The gel was stained in approximately 10 volumes of deglyoxylation buffer (50mM NaOH; 0.5µg/ml ethidium bromide) for 20 minutes and then in staining solution (10mM Tris-HCl

pH 7.5; 0.5µg/ml ethidium bromide) for 40 minutes. The gel was finally transferred to distilled water and viewed on a UV transilluminator.

3.2.12 Testing the translational activity of the RNA.

Purified RNA was tested in a typical *in vitro* translation set up, to see if the RNA had any translational activity. A full description of the method of *in vitro* translation is given in section 3.2.15. To test the RNA, a non-optimized reaction mixture was used, the one recommended by Amersham, and theoretically suitable for most RNAs. Activity was measured by visual comparison of tracks made on an SDS-PAGE gel autoradiograph, between the reaction containing the RNA sample, and the control reaction containing just water. The cpm were estimated to measure translational activity.

3.2.13 Oligo dT cellulose chromatography of total RNA.

Total RNA was prepared by the high SDS method of extraction as described in section 3.2.06. The integrity of the RNA was tested by denaturing gel electrophoresis as described in section 3.2.11. If there was no apparent degradation of RNA as shown by the gel, the sample was subjected to oligo(dT) cellulose chromatography to select for poly A+ RNA, i.e. the nuclear messenger RNA population. The method used is that described in Maniatis *et al.* (1982), and in Clemens (1984).

Oligo(dT) cellulose was equilibrated in sterile high salt loading buffer (0.50M NaCl; 1mM EDTA; 0.1% SDS; 10mM Tris-HCl, pH 7.5), and a 1-2ml column was poured in a sterile silicon coated pasteur pipette, plugged with silicon coated glass wool. The column was washed successively with water, 0.10M NaOH/5mM EDTA, and water again until the pH of the effluent was pH 7.0 or less. The column was then re-equilibrated by washing with at least 5 column volumes of loading buffer.

RNA was heated in a water bath to 65°C for 5 minutes, and an equal volume of 2-fold loading buffer was added. The RNA was allowed to cool to room temperature and then applied to the column. The effluent was collected in a sterile vessel and heated and cooled as before, and re-applied. The column was then washed with 10 volumes of loading buffer, until the A_{260} of the effluent was close to zero. A second wash was then applied to the column, using low salt loading buffer (0.10M NaCl; 1mM EDTA; 0.1% SDS; 10mM Tris-HCl, pH 7.5). 4 volumes of this wash were applied (this step is in the method of Maniatis *et al.*, 1982, but not in that of Clemens, 1984). The A+ RNA was eluted from the column by washing with 6 volumes of sterile low salt elution buffer (1mM EDTA; 0.05% SDS; 10mM Tris-HCl, pH 7.5) and collected in sterile siliconized eppendorf tubes. The RNA was made to 0.3M by adding a suitable amount of 3M ammonium acetate stock, and precipitated under ethanol as described in section 3.2.03

(ii). The RNA was habitually stored in pure sterile water at -80°C. The amount of A+ RNA produced was calculated from the absorbance (A_{260}) of 10µl of the sample in 0.5ml, as described in section 3.2.07.

3.2.14 Gel electrophoresis of A+ RNA.

A+ RNA was run on a 1% agarose gel in TBE buffer (0.089M Tris-borate; 0.089M boric acid; 0.002M EDTA). The purpose of this was to see the size distribution of the A+ RNA. The RNA was left in its native form, in spite of the fact that this distorts the running pattern, so that the secondary structure would allow incorporation of the dye and allow the RNA to be viewed. The gel was run for 3 hours at 60mA in TBE buffer. The gel mixture, running buffer and apparatus were sterilized by autoclaving and soaking in 0.1% DEP in ethanol to avoid degradation of the RNA by RNases. The gel was stained by adding a few drops of 10mg/ml ethidium bromide to the running buffer and destaining in sterile distilled water. The gel was viewed on a UV light box and photographed with a polaroid land camera with an orange filter, and a polaroid 665 black and white film.

3.2.15 *In vitro* translation of RNA.

(i) *In vitro* translation.

The *in vitro* translation system used was that supplied by Amersham, and the method used was that recommended by Amersham, but scaled down somewhat. A reaction mixture was set up containing 25µl of rabbit reticulocyte lysate, 2µl of 2.00M potassium acetate, 2µl of ^{35}S-methionine, 2µl of amino acid mix (minus L-methionine), to 35µl with RNA solution and water. The reaction mix was incubated at 30°C, and 2µl samples were taken out at 0, 5, 10, 15, 20 and 40 minutes.

(ii) TCA precipitation of labelled protein samples.

The samples were put into 0.5ml of 1.0M NaOH/10% H_2O_2 to decolourize them and heated to 37°C to hydrolyse the tRNA complexes. The samples were made to 1% SDS and 0.1% BSA and then made up to 2ml with 25% TCA. After an overnight precipitation at 0°C the samples were passed through pre-wetted Whatman GF/C discs and washed with 6 volumes of ice-cold 10% TCA. The discs were finally washed in ethanol, dried thoroughly, and put with at least 3ml of scintillant ('Optiphase') and counted in a Packard scintillation counter.

(iii) SDS-PAGE, and autoradiography of labelled proteins.

After 40 minutes the samples were either stored at -20°C, or run on an SDS-PAGE gel as described in section 2.2.07. The gels were generally stained and destained in Coomassie blue as in section 2.2.07 so that the markers could be clearly viewed, prior to PPO (2,5-diphenyloxazole) impregnation and

autoradiography. The destained gel was dehydrated in glacial acetic acid for 5 minutes, and then incubated for 2 hours in 20% w/v PPO in acetic acid. The gel was then washed in several changes of distilled water over the next hour before it was dried on a gel drier as in section 2.2.07. The dried and labelled gel was then put into an autoradiography cassette next to a piece of Kodak X-Ray film. The films were generally left to expose for a couple of days to a week, depending on the radioactivity of the proteins. The films were developed under a safe light, and generally had 5 minutes in Kodak X-Ray film developer, and 3 minutes in Kodak X-Ray film fix. The autoradiographs were rinsed and dried and analysed by eye or scanned on a Joyce Loebl densitometer.

(iv) Isoelectric focusing of labelled proteins.

Labelled proteins were treated as described in section 2.2.08 and run on a prepared IEF gel as described in that section. Due to the thinness and size of the gels PPO impregnation was not performed but instead the gel was hair-dryered onto a gel-bond (Pharmacia) backing and autoradiographed as described for SDS gels in the above section, 3.2.15 (iii).

3.2.16 Optimization of the *in vitro* translation reaction.

(i) The potassium ion concentration.

A number of solutions of differing K+ concentration were set up and used to alter the K+ concentration in six reactions. The method used was that recommended by Amersham International. The K+ concentration ranged from 85mM to 211mM, and the translations were carried out as described in section 3.2.15 (i). No magnesium ions were added. Duplicate reactions were sampled at 16 and 40 minutes. These samples were TCA precipitated as described in section 3.2.15 (ii), and used to compare the effects of K+ ion concentration on translation efficiency.

(ii) The magnesium ion concentration.

The method used to determine the K+ ion optimum was also used to determine the Mg++ optimum. Six reactions were set up, covering a range of Mg++ concentrations from 0 to 2.23mM. The potassium ion concentration was maintained at 162mM. The reactions were performed as in section 3.2.15 (i), but instead of samples being taken every 5 minutes duplicate reactions were sampled at 16 and 40 minute. These samples were TCA precipitated as described in section 3.2.15 (ii), and used to compare the effects on translation of Mg++ ion concentration.

(iii) The RNA concentration.

Six reactions were set up each containing the optimal amounts of K+ and Mg++ ions, but with differing amounts of total RNA. The total RNA concentrations in the reactions

ranged from 0.007 to 0.550 μg/μl. The reactions were carried out as described in section 3.2.15 (i). The six reactions were allowed to go on for 40 miniutes, and then a number of samples from each reaction mix were TCA precipitated as in section 3.2.15 (ii), and used to compare the effects on translation of RNA concentration.

3.2.17 *In vitro* translation of pepper fruit RNA using an optimized system.

Pepper fruit A+ and total RNA was translated *in vitro* as described in section 3.2.15 (i), except that the optimal concentrations of K+ and Mg++ ions (162mM and 0 respectively), and the optimal total RNA concentration of 0.14μg/μl were used (equivalent to an A+ RNA concentration of 0.0014 to 0.014 μg/μl). Samples were taken at particular times and TCA precipitated as described in section 3.2.15 (ii). An SDS-gel was also run of the labelled proteins as described in section 3.2.15 (iii) and an IEF gel of the products was also prepared, as described in section 3.2.15 (iv).

3.3.00 Results and Discussion.

3.3.01 RNA extraction.

The first method of extraction attempted was that recommended by P and S Biochemicals for the production of RNA suitable for use in the production of cDNA in their cDNA synthesis kit. The method uses a combination of guanidium thiocyanate extraction and centrifugation through a CsCl cushion. It is based on the method developed by Chirgwin *et al.* (1979), which in turn is developed from an earlier method using guanidium hydrochloride (Harding *et al.*, 1977). P and S suggest that the method should yield between 66 and 333 µg/g f wt of tissue (although they accept there is great variation from tissue to tissue), and Chirgwin *et al.* (1979) found the method produced a yield of 20 mg of RNA per g f wt from dog pancreas tissue. The main limitation of the method for pepper RNA was the centrifugation step of 130 000 *g* (37 000 r.p.m.) which had to be performed in a Beckman SW50.1 rotor, with a capacity of only 6 x 5 ml. The amount of total RNA that it was possible to extract by this method was extremely small due to the limits placed on the mass of starting tissue by the buffer volume. For reasons of economy the tube tops were not cut off as recommended by P and S, to leave the RNA pellet, but instead the RNA was resuspended in sterile TE buffer in the tube, and the tubes recycled by

soaking in 0.1% DEP in ethanol, and rinsing in sterile distilled water. This deviation from the recommended protocol probably contributed to the apparent failure of this method to produce translatable RNAs. The tube walls were in contact with a supernatant which was very rich in RNase. Any dilution of this RNase (such as during resuspension) would cause it to renature. RNase which was not removed by the DEP soak, or DEP which was not removed by the sterile water wash would interfere with the next batch of RNA, or react with the Tris base in the resuspension buffer (Berger, 1975; Leonard *et al.*, 1971; Chirgwin *et al.*, 1979) possibly rendering the RNA translationally inactive. The tubes were unsuitable for autoclaving and experienced a high failure rate anyway due to continuous exposure to high concentrations of GuSCN. Modifications were introduced into the method, specifically an increase in the mass of starting material per ml of GuSCN solution. The original mass of tissue per ml was 1 g per 10 ml, and this was increased to 1 g per 5 ml (Maniatis *et al.*, 1982; Cox and Goldberg, 1988), 1 g per 2 ml, and finally to 1 g per 1 ml (J.Beeching, personal communication). In spite of this the amount of RNA extracted remained very low and translatability did not improve. The yield of pepper leaf RNA extracted by the same method was approximately 4-fold higher than that of fruit. This may be due to the high level of hydration of fruit (leaves are about 16% dry weight, and fruit about 8%, data not shown). This method was rejected on

the grounds that the yields were too low to be useful, and in spite of a good appearance on multiwavelength scans the RNA would not translate in a cell free system.

The second method of RNA extraction was that recommended by Conover (1986). The method involves a phenol/chloroform extraction, followed by a CsCl gradient centrifugation in ethidium bromide to separate the DNA and RNA (Radloff *et al.*, 1967). Phenol and SDS alone cause loss of some RNAs and loss of some poly(A) tails. The loss is associated with certain proteins in polyribosomes, which enter the interphase between the phenolic and aqueous phases and cause loss of the RNA, or of the poly(A) tail. These losses are avoided if chloroform is included in the buffer/phenol mixture, or EDTA is included in the buffer to strip the proteins from the nucleic acids prior to extraction (Perry *et al.*, 1972). Both of these precautions are taken in the Conover (1986) method, and the interphase is also extracted twice to be certain that no RNAs are lost in it.

The yields obtained using this method were hugely variable and the purity of RNA extracted was the lowest of all the methods. A large amount of buffer to tissue was recommended (4 ml/1 mg) which presented problems with volume of phenolic extraction mixture. RNA taken from the gradients was found to be resistant to dissolution, and the intercalated ethidium bromide very difficult to remove. Extraction of

leaf RNA again proved much more successful than fruit RNA. RNA extracted in this way showed translational activity, but irregularly, and I decided to keep the basic method of phenol/chloroform extraction against an aqueous buffer but seek an improved method of separating RNA from DNA and polysaccharide.

The third method was that recommended by J.Beeching (Bath University) and also used to extract tomato RNA in Grierson *et al.* (1985). It involved a similar initial extraction to the method of Conover (1986), with the addition of a back extraction of the organic phase, and resuspension of total nucleic acid in 3 M sodium acetate, pH 5.5, to dissolve DNA and polysaccharides, which may inhibit translation, (Rattanapanone *et al.*, 1978) leaving the RNA as a pellet after centrifugation (Clemens, 1984). The final pellet was clear, gelatinous and easily dissolved. Yields were low but the RNA was very pure. Translational activity, however, was still elusive. The most likely point for RNases to attack RNA prepared by this method was during the initial minutes of the extraction when the frozen powder of the pepper tissue was allowed to thaw in extraction buffer. To eliminate possible degradation at this point the beginning of Conover's (1986) method was adopted, and the tissue dropped directly onto the swirling blades of the homogenizer, into cold, phenol-saturated buffer. This final method resulted in low (but fairly consistent) yields,

Figure 1. A comparison of the yield and purity of pepper fruit RNA extracted by four different methods.

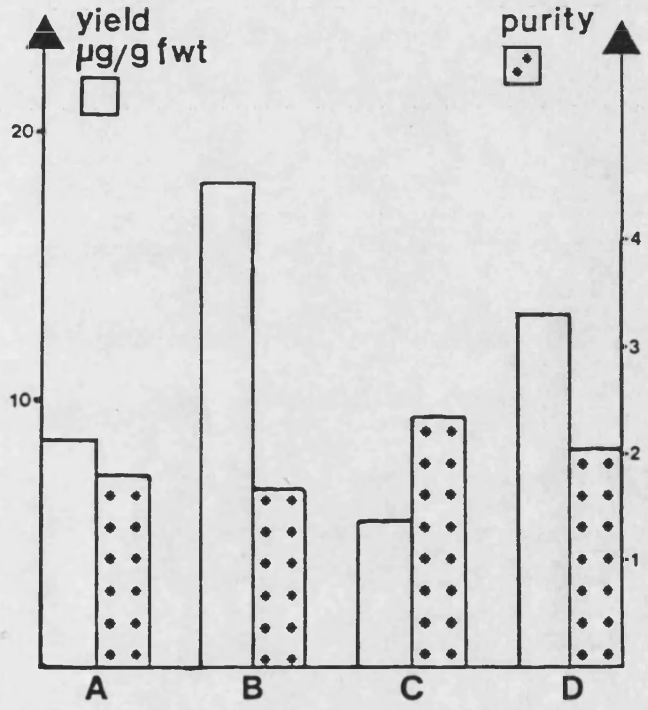


Figure 1. RNA was extracted by A, the guanidium isothiocyanate method; B, phenol extraction and caesium chloride centrifugation; C, the high SDS method; and D, a method developed by combining B and C. The methodology is described in sections 3.2.03 to 3.2.06. Yield was calculated as µg RNA per g fresh weight (g f wt) of fruit. Purity is represented as the Abs_{260}/Abs_{280} ratio.

Figure 2. Scanning RNA samples between wavelengths 200-300 nm to demonstrate purity.

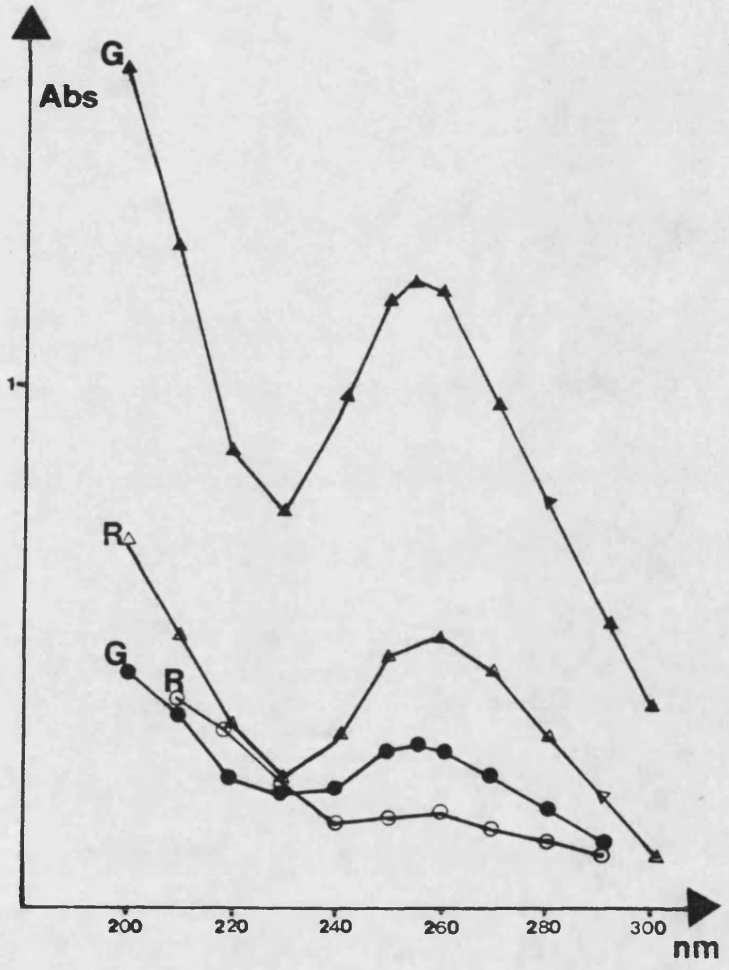


Figure 2. RNA was extracted by one of the methods described in sections 3.2.03-3.2.06. An aliquot was dissolved in water in a quartz cuvette of 1 cm light path. The sample was scanned against a zero of water and the absorbances at each wavelength recorded. Filled triangles, green fruit RNA extracted by the phenol/caesium chloride method; open triangles, red fruit RNA extracted by the phenol/caesium chloride method; filled circles, green fruit RNA extracted by the guanidium isothiocyanate method; open circles, red fruit RNA extracted by the guanidium isothiocyanate method.

Figure 3. Tube gel electrophoresis of pepper fruit RNA.

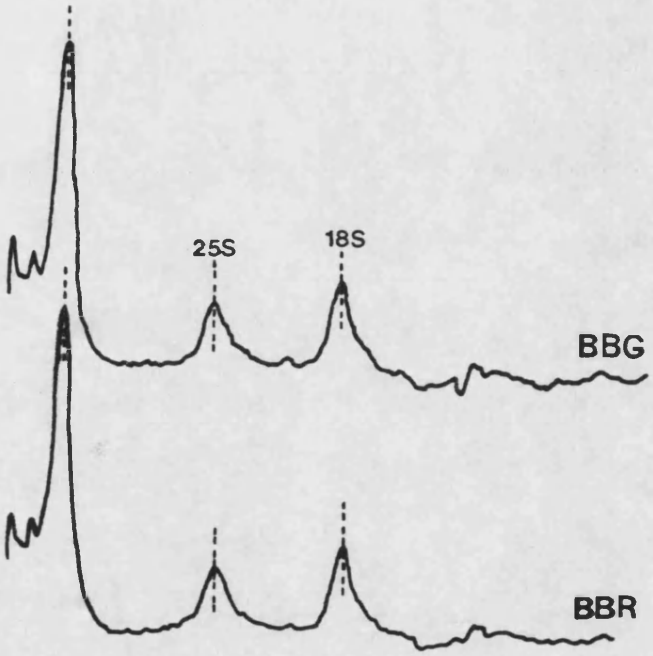


Figure 3. RNA was loaded onto pre-run polyacrylamide gels, prepared in quartz tubes, and run as described in section 3.2.10. The gels were scanned against blanks of tube gels with no RNA. Interpretation of the results was as in Grierson (1982) and Bathgate *et al.* (1985). BBG, Bellboy green fruit RNA; BBR, Bellboy red RNA.

Figure 4. Denaturing gel electrophoresis of pepper fruit RNA.

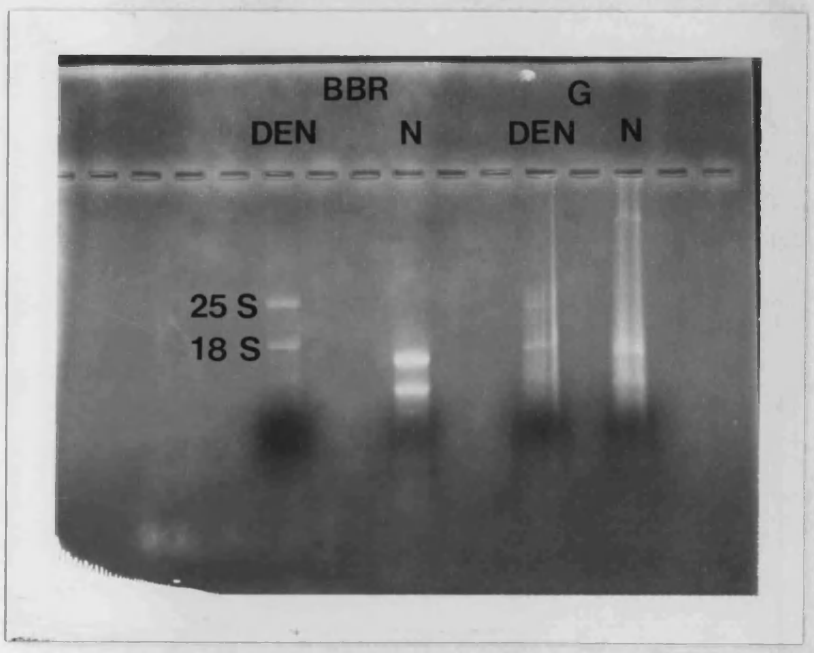


Figure 4. Pepper fruit RNA was extracted as described in sections 3.2.03 to 3.2.06. It was then treated as described in section 3.2.11 prior to being run on a sterile 1.1% agarose gel with constant recirculation of buffer. The gel was stained and destained as described, viewed on a UV transilluminator, and photographed with a Polaroid Land camera. Interpretation of the results was helped by the experiment described in Appendix A, where the RNA was electrophoresed against SP6 RNA and sized.

acceptable purity, and most importantly, a high level of translation in a non-optimized *in vitro* translation system (Figures 1 and 5).

3.3.02 Yield and purity.

The yields of RNA from pepper fruit tissue are very low compared with some reports in the literature (3 mg/g f wt of leaf, Cashmore, 1982; 66-333 µg/g f wt of 'most tissue', P and S Biochemicals Ltd). Fruit tissue seems to produce low and variable amounts of total RNA per g fresh weight. For example, in the avocado, Christoffersen *et al.* (1982) report yields of total RNA of 28-56 µg/g f wt. In the tomato, Piechulla *et al.* (1986) report yields which vary from 10 to 1000 µg/g f wt. The yield of pepper RNA in this work varies between 5 and 25 µg/g f wt. This seems low but is in accordance with the results reported above. Possible reasons for the low yield include the high level of hydration of the fruit; low levels of endogenous RNA; and loss of the RNA in some way during extraction. Fruits containing high levels of polysaccharide may have apparently lower levels of RNA because the polysaccharide may hold the RNA in solution and cause its loss during extraction. The large volumes of tissue used probably resulted in increasing inefficiency of the extraction method, and exaggerated the poorness of the yield.

Purity was measured for each sample as the ratio between A_{260} and A_{280} (Figure 1). A pure sample of RNA has a ratio of 2. Other ways of demonstrating purity of an RNA sample were investigated including scanning (Figure 2), tube gel electrophoresis (Figure 3) and denaturing gel electrophoresis (Hatfield Polytechnic, Nucleic Acids Workshop) (Figure 4). Purity became secondary to efficiency of translation, so this set of experiments was discontinued except that denaturing gel electrophoresis was used to demonstrate the purity and intactness of a translating RNA prior to selection on oligo(dT) cellulose. The ability of extracted RNA to direct translation of proteins in a cell free system is summarized in Figure 5 for each of the methods of extraction investigated.

3.3.03 Oligo(dT) cellulose chromatography.

Total RNA was subjected to oligo(dT) cellulose chromatography to separate the nuclear A+ RNA from the (plastid) A- RNA (Wheeler and Hartley, 1975). Oligo(dT) cellulose was obtained from Amersham, prepared as described in Gilham (1964). The procedure was carried out according to Maniatis *et al.* (1982) and Aviv and Leder (1972). Various problems arose associated with the technique, such as blocked columns, the appearance of cellulose in the eluate and the slow and cumbersome measurement of absorbance at 260 nm of individual fractions. In spite of these drawbacks the

method proved reliable. Problems encountered with blocked columns were solved by forcing solutions through the column under pressure, and eventually by omitting SDS from the buffers (SDS precipitates out at lower temperatures), which had the added advantage of ensuring that no SDS could contaminate the eluate. A typical elution profile is given in Figure 6. Further adaptions to the method were made in an attempt to improve the translational activity of the RNA extracted, including the omission of SDS from the column buffers, repeated ethanol precipitation of the RNA to remove traces of impurities, and prolonged desiccation of the RNA under vacuum to remove traces of ethanol.

The A+ RNA produced by oligo(dT) cellulose chromatography was subjected to gel electrophoresis (Figure 7). The appearance on the gel was that of a smear, indicating the presence of some large, variably sized species which implied that the RNA was relatively intact.

3.3.04 Optimization of the *in vitro* translation reaction.

The cell-free translation system chosen for the translation of pepper fruit RNAs was rabbit reticulocyte lysate, obtained from Amersham International. The lysate was depleted in amino acids so that the amino acid concentrations could be set by the addition of exogenous

Figure 5. *In vitro* translation of total RNA extracted by the different methods.

Method of extraction	Translation
GuSCN	-
Phenol/CsCl	+/-
High SDS	-
Combined method	++

Figure 5. A standard non-optimized *in vitro* translation reaction was set up, using total RNA extracted by one of the four methods described in sections 3.2.03 to 3.2.06. The translatability of the RNA was determined by visual comparison of labelled gel tracks produced on an autoradiograph of an SDS-PAGE gel of the labelled translation products. Those RNAs which were not appreciably different from the control were designated minus (-) and those that showed high contrast (translatability) were designated plus (+). A typical reaction mixture contained 35 µl of lysate, 2.5 µl of amino acid solution (minus methionine), 2.5 µl of ^{35}S[methionine], 2.5 µl of 20 mM K^+ solution, 10 µl of RNA (20 to 30 µg) in sterile water. The reactions were left at 30°C for 40 min and a sample run on a 10% polyacrylamide gel as described in section 3.2.15(iii).

Figure 6. Oligo (dT) cellulose chromatography of total pepper fruit RNA.

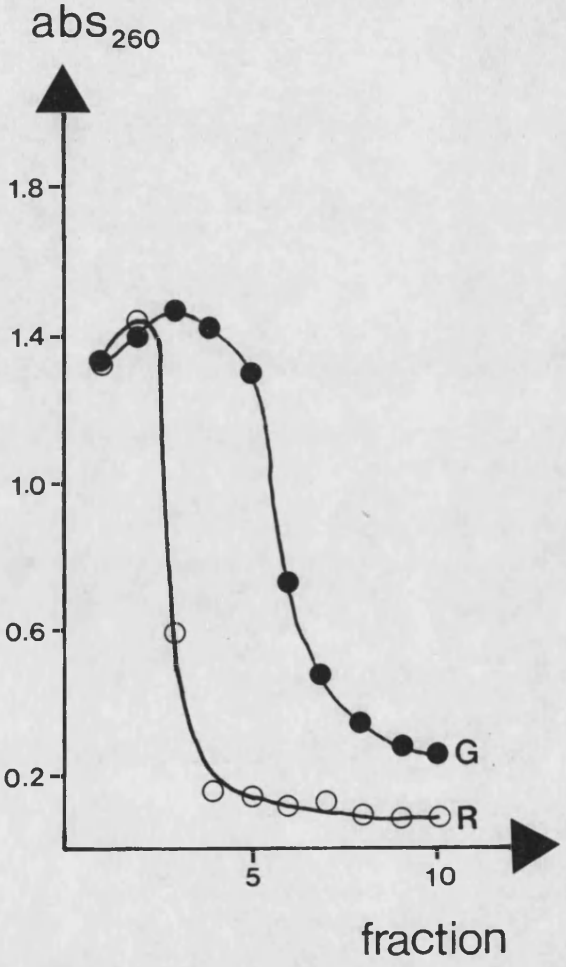


Figure 6. Total RNA was treated as described in section 3.2.13 and loaded onto a pre-equilibrated oligo(dT) cellulose column as described. The non-polyadenylated RNA was washed from the column with loading buffer and the A_{260} of each 1 ml fraction measured against a buffer blank. The graph shows elution profiles of red (open circles) and green (filled circles) pepper fruit RNA.

Figure 7. Denaturing gel electrophoresis of A+ RNA.

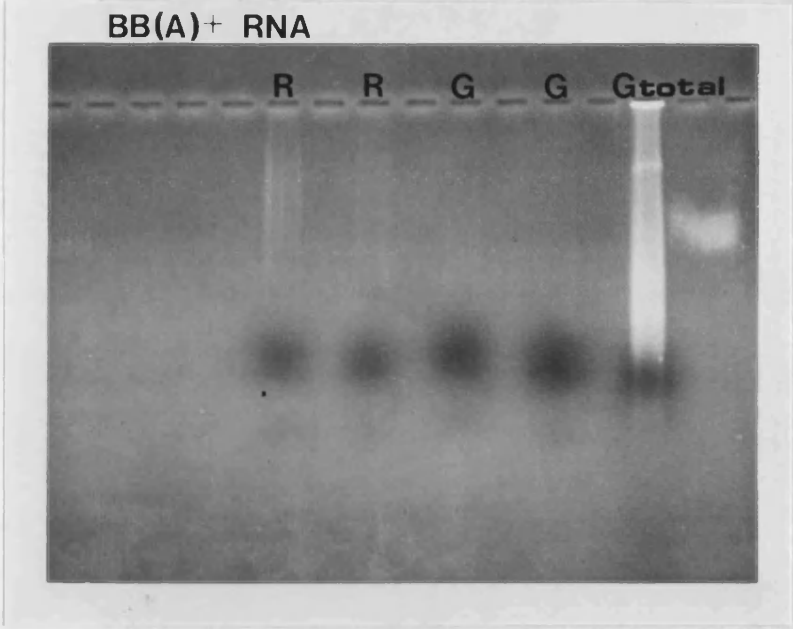
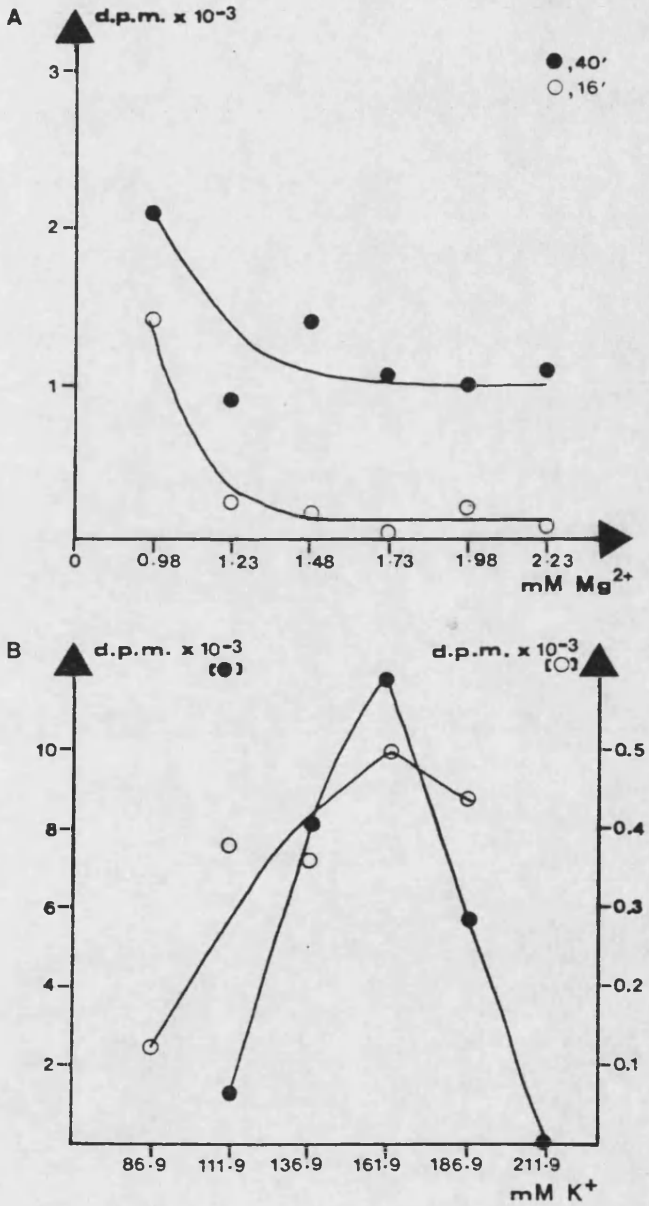


Figure 7. Total RNA was subjected to oligo (dT) cellulose chromatography. The resulting polyadenylated RNA was pooled and concentrated by ethanol precipitation, dried and dissolved in buffer prior to being run on a sterile 1.1% agarose gel. The gel was run, stained and destained as described in section 3.2.14.

material, and also depleted in potassium and magnesium ions so that the optimal concentrations of these ions for pepper fruit RNA could be determined. The lysate is previously treated with micrococcal nuclease to remove endogenous RNA so that translation is exogenous RNA dependent (Pelham and Jackson, 1976). Optimization is recommended as addition of non-optimal amounts of K^+ and Mg^{2+} may cause low incorporation of label, or premature truncation of protein chains (Amersham Product Information). The optimal concentration of RNA required was also determined. Experiments were carried out according to the protocol recommended by Amersham, and the results are shown in Figure 8. Results agreed with those determined for TMV RNA by Amersham, that no additional Mg^{2+} should be added to the lysate, and that the total concentration of K^+ is optimal at about 160 mM. Total RNA concentration was found to be optimal at 140 µg/ml of reaction mixture (Figure 8C). Pelham and Jackson (1976) report optimal incorporation of label in a globin mRNA-dependent lysate at 20 µg/ml. If it is assumed that mRNA comprises between 1 and 10% of total RNA it can be seen that this work produces a value of about 14 µg/ml, which is comparable to the Pelham and Jackson result. Concentrations of RNA higher than the optimum resulted in decreased label incorporation, in fact, addition of a low-translating pepper RNA to a high-translating TMV RNA reaction resulted in drastic loss of TMV-directed

Figure 8. Optimization of the *in vitro* translation reaction.



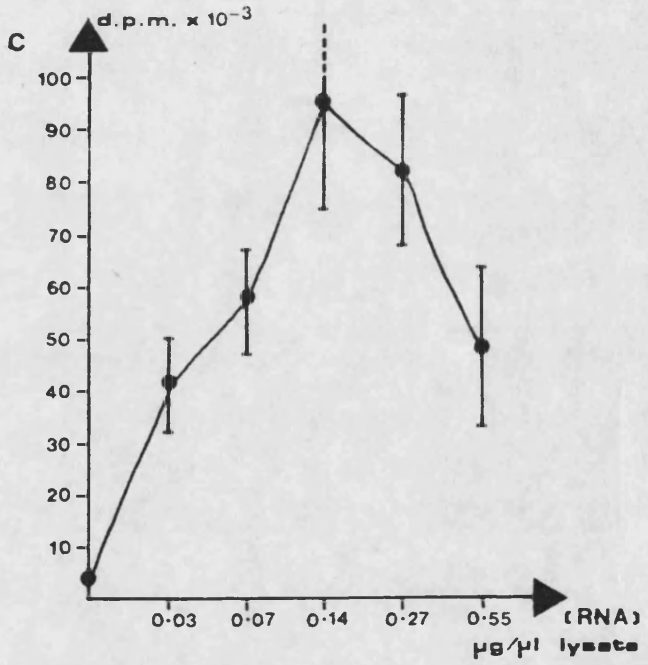
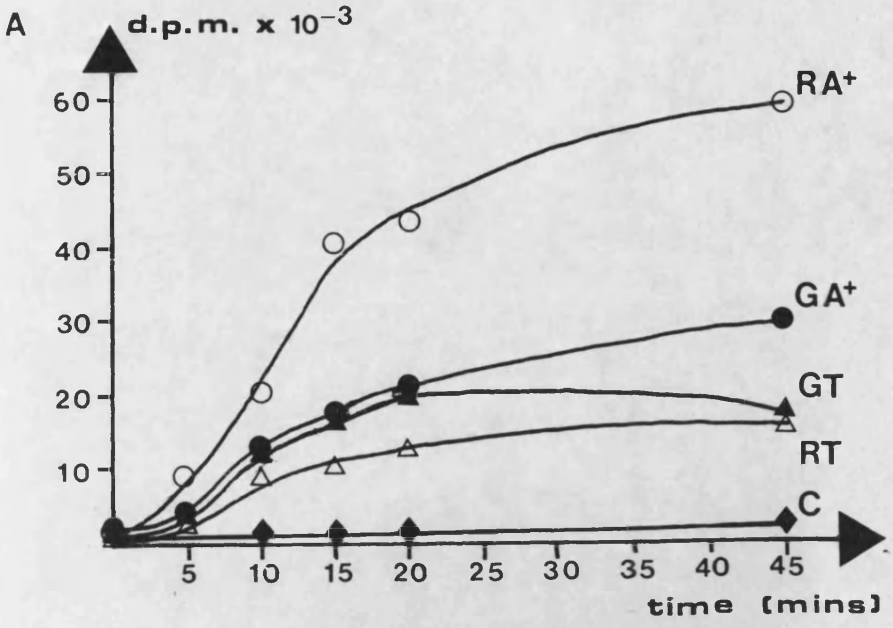
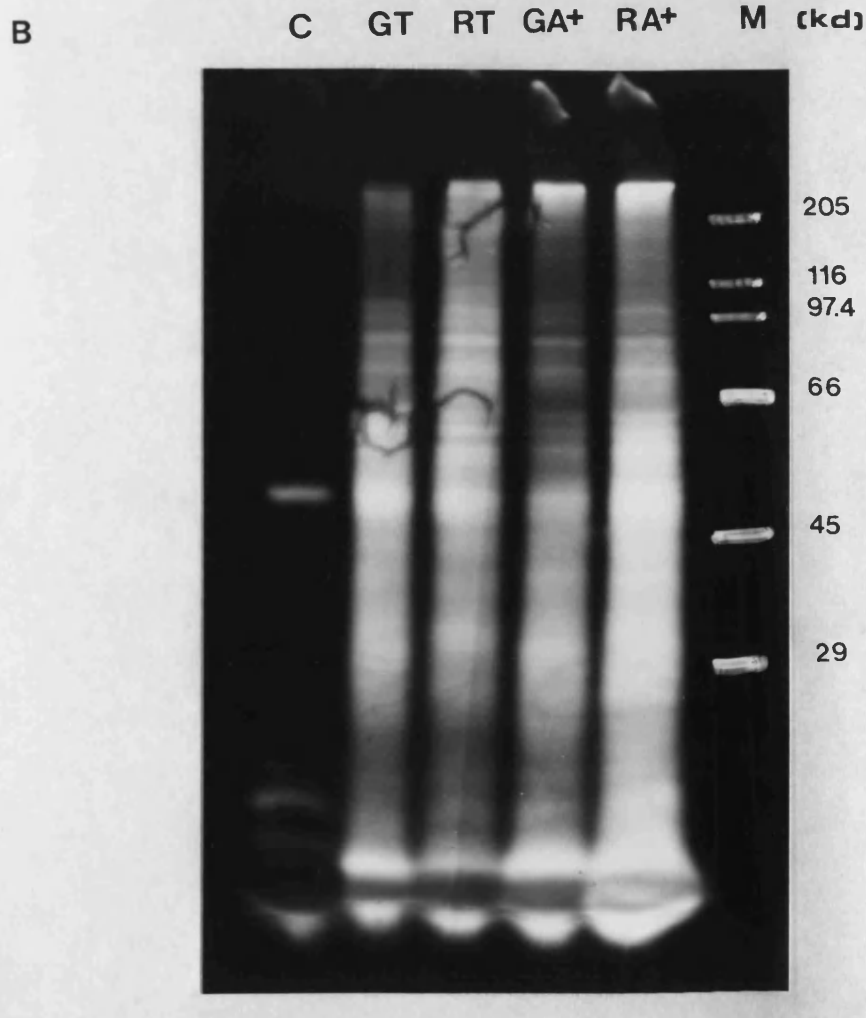


Figure 8. The Mg^{2+} and K^{+} concentrations (A and B respectively) were optimized by altering one ion concentration and keeping the other constant as described in section 3.2.16. The total RNA optimum (C) was determined using the Mg^{2+} and K^{+} optimized system.

Figure 9. *In vitro* translation of pepper fruit RNA.





C

Bellboy green RNA		Bellboy red RNA	
A+	Total	A+	Total
			240
190	190	190	
180	180	180	
		160	
140	140	140	
		136*	
		130	
120		120	120
110	110		
		106	106
98	98	98	98
88*	88	88*	88
		82	
78*	78	78*	78
		73	
64*	64	64*	64
60*	60	60*	60
54*	54	54*	54
		49*	
46*	46	46*	46
	40		40
		43	
		38	
37	37	37	37
		32	32
31	31		
28		28*	
24	24		
		23	
22	22	22*	22

Figure 9. Part A is a graph showing the time-course of incorporation of labelled amino acids into polypeptides in an RNA-directed *in vitro* translation system. Open circles, red fruit A+ RNA; filled circles, green fruit A+ RNA; open triangles, red total RNA; filled triangles, green total RNA; diamonds, no RNA control. Part B is an autoradiograph produced by exposing an SDS-PAGE gel of *in vitro* labelled proteins to X-ray film. The lanes are labelled as follows; C, control; GT, green fruit total RNA; RT, red fruit total RNA; GA+, green A+ RNA; RA+, red A+ RNA; M (kd), m wt markers. Part C is a table, giving the m wts of the *in vitro*-produced polypeptides in kD.

incorporation (data not shown). A possible explanation of this is that some inhibitory substance precipitated with, and purified with the pepper RNA. Possibilities include phenol, SDS and ethanol, hence the modifications described in the previous section (section 3.3.03) to remove possible contaminants.

3.3.05 *In vitro* translation of pepper RNA.

(i) Analysis of results.

The aims of *in vitro* translation of RNA from ripe and unripe fruit of *C.annuum* var. Bellboy were as follows: to establish whether variation of the RNA population took place during the ripening process; whether there were RNAs present in ripe fruit that were absent from unripe fruit (i.e. ripe fruit specific RNAs); to estimate the apparent m wts of any such *in vitro* translated products; to correlate these m wts with the m wts of plastid proteins determined in the previous chapter; to compare the m wts of the *in vitro* translated products with those of the products reported in the literature and to judge the biological viability and intactness of the RNA sample, and hence its suitability for use as a template in the construction of a cDNA library.

RNA was extracted as described in section 3.2.06, assessed for purity and intactness by denaturing gel electrophoresis

as described in section 3.2.11, and if mRNA was required, subjected to oligo(dT) cellulose chromatography. The RNA was used to direct *in vitro* translation in an optimized rabbit reticulocyte lysate system as described, and the products subjected to SDS-PAGE and autoradiography. A time course of the reaction is presented in Figure 9A, an autoradiograph in Figure 9B and analysis of the products in Figure 9C.

C.annuum var. Bellboy was the only variety successfully studied. Lito and Golden Star were initially included in the study but problems with technique encouraged a simplification of approach. The proposed cDNA library was to made from mRNA isolated from ripe Bellboy fruit, and it was therefore paramount to establish procedures for the extraction and translation of Bellboy fruit mRNA. Lito and Golden Star RNAs were to be used later in the work to aid characterization of a ripening associated cDNA clone, and a comparative study between ripe and unripe fruit for Lito and Golden Star was seen as being related to that work. The results presented in Figure 9C can be summarized using the classification described by Grierson *et al.* (1985). (i) Products present only or in greatly increased amounts in unripe fruit; (ii) products present in both ripe and unripe fruit; (iii) products present only or in greatly increased amounts in ripe fruit. For *C.annuum* var. Bellboy m wts of the *in vitro* translation products in the three classes are as follows (major products are marked with an asterisk):

(i) 110, 31 and 24 kD.

(ii) 190, 180, 140, 120, 98, 88*, 78*, 64*, 60*, 54*, 46*, 37 and 22 kD.

(iii) 160, 136*, 130, 106, 82, 73, 49*, 43, 38, 32, 28* and 23 kD.

Regarding the *in vitro* profiles, most products present in the total RNA translation profile are also represented in the A+ profile, and additional products are present in the A+ track implicating the A+ RNA as the seat of control. One product of m wt 40 kD is present in the 'total RNA' track of both red and green fruit, but is absent from the A+ track. This RNA is apparently A-, either an organellar RNA or a nuclear RNA lacking a poly(A) tail.

(ii) Criticism of the method.

The rabbit reticulocyte lysate system used was treated with micrococcal nuclease to inactivate endogenous RNAs and fractionated to remove amino acids to make subsequent translation dependent on added RNA and amino acids, one of which may be radioactively labelled. The lysate was developed by Pelham and Jackson (1976) and is described as having very efficient utilization of added RNA with high translational ability, low background incorporation, and the ability to synthesize large protein products with a low yield of truncated protein. However, it has been shown that

lysate mixtures treated with micrococcal nuclease retain RNA fragments, some of which contain initiation sites (Clemens, 1984). These fragments compete with the exogenous RNA for ribosome binding, and weakly initiating exogenous RNAs may not compete efficiently. The lysate may also be inhibited by low concentrations of double stranded RNA and oxidized thiol compounds. A further limitation of the method is the use of a single labelled amino acid (in this case methionine) as polypeptides with low levels of that amino acid will give an accordingly weak signal. The emphasis of bands in an *in vitro* translation experiment may not, therefore, reflect the *in vivo* situation accurately. The effect of viral infection of the experimental tissue was demonstrated by Grierson *et al.* (1985), in tomato infected with TMV. The presence of viral RNA drastically changed the *in vitro* translation profile of the infected plant. The products of interest here are those which are different between red and green fruits, and the assumption was made that plants would be equally infected if infected at all, and the products of interest would therefore be *bona fide*. Monitoring the effect and extent of viral infection in pepper fruit with regard to translation studies would be a valid related project.

Successful *in vitro* translation was initially very elusive due to the low quality of RNA extracted. Translation in a non-optimized system was routinely performed on extracted RNA as a means of judging RNA quality; and alterations in

the methods of extraction and oligo(dT) cellulose chromatography were monitored by this method (the alterations and adaptations are described in sections 3.3.01 and 3.3.04). A problem with this approach is the expense associated with purchasing commercially available lysate, and in retrospect it may have been preferable to develop the technique of making the lysate myself. Also a great deal of time was wasted with a batch of non-viable lysate, after which lysate was routinely tested with Amersham's TMV RNA before any assumptions were made about its activity, involving more expense.

(iii) Discussion of the results.

The m wts of the *in vitro* translation products can be compared to those determined by SDS-PAGE of plastid proteins. Although the plastid is the site of much metabolic activity, particularly during ripening, the vast majority of plastid proteins are encoded in nuclear genes and synthesized by cytosolic ribosomes. This is especially true of *C.annuum* chromoplasts as recent work suggests cessation of plastidic protein synthesis (Carde *et al.*, 1988). A protein destined for the plastid is typically synthesized as a larger precursor molecule, with a plastid 'targeting sequence' of between 2 and 16 kD (usually between 5 and 8 kD) (Ellis and Robinson, 1987; Keegstra and Bauerle, 1988). This presequence, or transit peptide, contains information

necessary to direct transport of the attached peptide into its ultimate plastidic location. Transport is believed to be carried out by binding of the presequence to a receptor on the plastid outer membrane, and subsequent transition of the transit and main protein to the targeted compartment. The transit peptide is then cleaved off by proteases. Most work on the import of proteins into plastids has been done with chloroplasts, but it is assumed that chromoplasts function similarly. Due to the m wt of the presumed transit peptide (between 2 and 16 kD), it is not possible to compare directly the m wts of the SDS-PAGE resolved plastid proteins, and the *in vitro* translation products. Correlations are possible if it is assumed that the *in vitro* translation product is between 2 and 16 kD larger than the possible plastid located protein, and that the pattern of expression of the plastid and *in vitro* translated product will be similar. The different classes of product (as defined earlier in this section) which correlate in these ways follow. The m wt of the presumed precursor protein is given first and the possible m wts of plastid products given in parentheses.

Class i (unripe fruit only):

31 kD (29, 26, 22* and 16 kD).

24 kD (26, 22* and 16 kD).

Class ii (both ripe and unripe fruit):

120 kD (105 kD).

98 kD (93 kD).

88*kD (71 kD).

78*kD (71, 66, and 61 kD).

64*kD (61 kD).

22 kD (11 kD).

Class iii (ripe fruit only):

160 kD (148 kD).

130 kD (116 kD).

106 kD (99 and 87 kD).

82 kD (75 kD).

73 kD (58 kD).

49*kD (47, 42 and 33* kD).

43 kD (33* and 27 kD).

38 kD (33*, 27 and 25 kD).

32 kD (27 and 25 kD).

28*kD (25 and 14 kD).

23 kD (14 kD).

From this analysis it is possible to conclude that several of the *in vitro* translated products are possible larger precursors of proteins found in the plastids. Of the ripening associated proteins, the possible correlation between the 33* kD plastid protein and the 49* kD *in vitro* translation product is strengthened by their appearances as major products of both the *in vitro* translation represented

mRNA population, and in possible ripening associated chromoplast proteins.

Comparison of the m wts of pepper *in vitro* translation products with m wts of *in vitro* translation products reported in the literature reveals five pepper products with m wts similar to those reported. These are pepper products of m wt 82 kD (tomato, 80 kD, Grierson *et al.*, 1985; avocado, 80 kD, Christoffersen *et al.*, 1982); 49 kD (tomato, 48 kD, Grierson *et al.*, 1985); 43 kD (tomato, 44 kD, Grierson *et al.*, 1985); 38 kD (tomato, 35 kD, Grierson *et al.*, 1985; avocado, 36 kD, Christoffersen *et al.*, 1982); and 23 kD (tomato, 20 kD, Grierson *et al.*, 1985).

Regarding comparison of the 49 kD protein produced by *in vitro* translation, a product of similar m wt (48 kD) was concluded to be an unmodified precursor of the softening enzyme polygalacturonase by Grierson *et al.* (1985). A 43 kD protein was described as normal sized polygalacturonase from SDS-PAGE of plastid proteins (Handa *et al.*, 1983). Tomato polygalacturonase is also described as being of m wt 46 kD (Grierson *et al.*, 1985; Rattanapanone *et al.*, 1978; Lee *et al.*, 1987). Similarity with a pepper ripening associated protein m wt may be coincidental. Further experiments would be required to verify that polygalacturonase is active in ripening pepper fruit; the lack of softness of the ripe fruit seems to suggest that it is not significant.

Some interesting speculations may be made from an assessment of the results presented here. Some proteins are found when polypeptide content is analysed directly by SDS-PAGE, but are not represented in the mRNA population as possible precursors, as demonstrated by *in vitro* translation (e.g. the 160 kD product of both ripe and unripe Bellboy fruit plastids, and the 56 kD product [large s.u. of Rubisco] of fruit chloroplasts). These proteins may be synthesized within the plastid, and so possess A- RNA which would not have been selected on the oligo(dT) column. Further reasons for non-representation in the *in vitro* profile include the limitations of the lysate system, strongly initiating RNAs may be translated 40-60 times during an incubation and weakly initiating species have to compete, not necessarily efficiently (Clemens, 1984). Low methionine content may also be responsible for a weak signal. Another possibility is that stable and long-lived polypeptides may be produced from low levels of RNA, producing an apparent discrepancy between the two methods of investigation. The two prominent ripening products of purified plastids have approximate m wts of 33 and 53 kD. Of these two only the 33 kD product appears to be represented in the *in vitro* translation profile (at 49, 43 or 48* kD) as a ripe fruit specific product, the 53 kD polypeptide is represented in both the red and green profiles at 64 or 60 kD. A possible explanation of this is that the RNA is constitutively expressed, but is not normally translated or maintained *in vivo* in the green

fruit. This implies activation of translation as a regulatory mechanism in fruit ripening. Translational control is established as a method by which the gene expression of plastids is regulated (Deng and Gruissem, 1988). It is possible that factors in the ripening fruit may promote translation or maintenance of the 53 kD nuclear product in a similar way to the plastid dependent expression of the nuclear encoded LHCP II protein (Batschauer *et al.*, 1986). Another possibility is that the 53 kD products in red and green fruit are different polypeptides and the green 53 kD product is reflected poorly by SDS-PAGE and Coomassie blue staining. It is unlikely that the 53 kD product is synthesized in the chromoplasts, as these organelles have been shown to be probably non-active in protein synthesis (Carde *et al.*, 1988).

Other polypeptides are found in the *in vitro* translation products but are absent from plastid profiles. These products probably represent cytoplasmic rather than plastid located proteins. Other explanations include lack of response of the product to the staining method used to create the plastid profiles, or the presence of RNAs which are not translated (or maintained) in significant quantities of protein *in vivo*. Some nuclear and plastid genes are transcribed according to a diurnal rhythm, as has been described for the LHCP II mRNA of tomato (Piechulla and Gruissem, 1987), and therefore it is possible to extract the

proteins when the quantity of a particular species is at an unusually high or low level.

To conclude: the search for mRNAs associated with ripening fruit yielded several *in vitro* translation products, many of which could be possible precursors of chromoplast proteins. Therefore the aims of these experiments which are outlined at the start of this section have largely been met. The next stage was to use the results and techniques so far determined for the next phase of experiments, the construction and analysis of a cDNA library.

Chapter 4. Creation of a ripe fruit derived cDNA library, and identification of ripening related clones.

4.1.00 Introduction.

This chapter describes an attempt to identify ripening specific cDNA by differential colony hybridization. Bellboy red fruit RNA was made into cDNA using reverse transcriptase and RNaseH (Gubler and Hoffman, 1983). The cDNA was cloned into the ampicillin gene of pBR322 by dC-tailing the cDNA and annealing to commercially obtained dG-tailed plasmid. Transformed and recombinant cells were identified by their combined tetracycline (tet) resistance and ampicillin (amp) susceptibility. These recombinants were screened in duplicate with either ripe or unripe fruit labelled cDNA. Those colonies hybridizing more strongly, or entirely, to the red cDNA were selected for further study as this indicated possible ripening specificity. The sizes of the inserts were looked at by making minipreparations (separation of the plasmid DNA from the bacterial chromosome and cell debris, digestion of the plasmid and analysis by agarose gel electrophoresis). Four clones were of sufficient size to be interesting and maxipreparations were made of these in order to increase the quantity of insert DNA. The eventual aim of isolation of a ripening specific cDNA clone(s) was to investigate its expression during ripening,

both in Bellboy, and Lito and Golden Star fruit. Once cDNA inserts of suitable size were identified, and after their ripening associated nature and relatedness to each other had been established, the investigation would have taken one of three theoretical directions. These directions involve: study of the spatial and temporal expression of the cDNA gene; regulation of expression of that gene; and a study of the gene product. The approaches are not isolated, and techniques valid for one may also be applicable to another.

The study of temporal and spatial gene expression was to involve a study of levels of clone-complementary RNA during ripening in normal and mutant fruit, by extracting total RNA and dot blotting on nitrocellulose to a labelled cDNA. This type of study has been performed on tomato by Mansson *et al.* (1985). The levels of ripening related cDNA RNAs were assessed by hybridization to total RNA from normal and ripening mutant fruit. Thus, the absence or presence of the message in the ripe and unripe, and mutant fruits was ascertained. Once a pattern of expression levels in normal and mutant fruit, and other plant tissues, had been established, it would be possible to vary certain environmental factors in an effort to assess the effect on gene expression. Ethylene levels, temperature, carbon dioxide concentration, daylength or levels of plant hormones such as auxin are factors which may merit investigation, e.g. in the non-climacteric strawberry, declining levels of

auxin in the achenes appear to modulate the rate of ripening which is independent of inhibitors of ethylene synthesis and action (Given *et al.*, 1988).

A study of the regulation of the ripening associated gene could be approached in the following ways. The first approach involves sequencing of the cDNA insert. From the 5' sequences possible regulatory regions could be identified, for example the cap site. The cDNA could be used to isolate the genomic complementary sequence and the 5' region of this further investigated for regulatory sequences (such as the plant equivalent of the animal TATA box [TC/GTATAT/AA$_{1-3}$C/TA], and the further upstream AGGA or CAAT box [Grierson and Covey, 1984]) and the 3' region studied for the presence of translation stop sequences and poly A addition sequences (Grierson and Covey, 1984). A comparison of the cDNA and genomic sequences would allow study of the presence of introns, and RNA splice sites. The regulatory effects of the upstream sequences could be investigated using a hybrid gene construct, with a marker gene such as chloramphenicol acetyl transferase (CAT) or by looking at the behaviour of the gene in a transgenic plant. Effective regulatory regions could be identified by mutational analysis or sequential deletion, and their mode of action studied. Goldberg (1986) isolated gene fragments responsible for the regulation of soybean lectin genes and showed binding by protein factors specific for the tissue in which the lectin genes were expressed

(embryo). These factors were thought to activate transcription.

The gene product could be primarily characterized by hybrid release or hybrid arrest *in vitro* translation. These techniques were used by Weinard *et al.* (1979) in the identification of cDNAs specific to developing maize endosperm, and by Mignery *et al.* (1984) in the identification of patatin from developing potato tuber. Antisera to the protein could be raised in two ways: by purification of the protein to yield sufficient quantity for the production of antisera; or by the construction of a synthetic polypeptide, the amino acid sequence of which could be deduced from the cDNA sequence. The antibody so raised could be used to locate the protein *in situ*, or to observe the *in vitro* effects of 'eliminating' the protein. A comparison of the amino acid sequences of the native polypeptide and the amino acid sequence deduced from the cDNA sequence may reveal the presence of a signal peptide. Hattori *et al.* (1985) used the cDNA sequence of a sweet potato tuber protein (sporamin) to study the presence and sequence of a signal peptide. A search of protein or nucleotide databases may reveal a similar protein or nucleotide sequence, which would allow the protein or cDNA to be categorized, and perhaps help with elucidation of the function of the protein. Other clues to the protein function may be derived from its *in situ* location, hydropathy plots,

and glycosylation sites in the primary sequence. Addition of purified protein to pepper tissue *in vitro* and subsequent analysis of the effects chromatographically or ultrastructurally, or by observing the effects on protein or RNA populations may lead to elucidation of possible functions of the protein. This type of approach was utilized in determining the function of isolated polygalacturonase in tomato (Themmen *et al.*, 1982). Purified polygalacturonase was added to tomato fruit parenchyma tissue *in vitro*, and the degrading effect on the cell walls observed ultrastructurally.

Function of the gene product may also be investigated using antisense RNA. Antisense RNA binds to the messenger RNA, perhaps at a regulatory region, and prevents translation of that RNA. The phenotypic effects of inhibition of expression may be observable. An antisense RNA of a *Drosophila* segmentation gene was produced using the SP6 vector *in vitro* transcription system to transcribe the non-sense strand of the gene. The resulting RNA was injected into genetically normal embryos, which then appeared phenotypically to lack the gene (Rosenberg *et al.*, 1985). Hamilton *et al.* (1990) used the plant transformation abilities of *Agrobacterium tumefaciens* to transfer an antisense cDNA construct of a ripening related gene into tomato. Expression of the antisense RNA was monitored by Northern blotting of leaf RNA to probes specific for the antisense strand and the

phenotypic effects of the antisense RNA (reduction in ethylene levels) observed experimentally.

The work presented in this chapter comprises experiments designed to create a ripening related cDNA library derived from pepper fruit, and the isolation of those clones likely to be ripening specific.

4.2.00 Materials and Methods.

4.2.01 RNA and cells.

RNA was extracted as described in section 3.2.06 and tested for integrity by denaturing gel electrophoresis as described in section 3.2.11. The intact total RNA was then subjected to oligo(dT) cellulose chromatography as described in section 3.2.13, and optimized *in vitro* translation as described in section 3.2.15. If the RNA gave high molecular weight translation products it was used to produce cDNA.

The cell line used was *E.coli* strain HB101, recommended and donated by Dr J.Beeching (Bath University). The storage and growth curves for HB101 are given in Appendix C.

4.2.02 Chemicals.

The oligo(dT_{12-18}), the nucleotides, RNasin and reverse transcriptase all came from Amersham. The BSA, RNase H (*E.coli*), [^{32}P]dATP, DNA polymerase 1, restriction enzymes, [^{3}H] dCTP, terminal transferase and antibiotics were also from Amersham. dG tailed pBR322 was obtained from BRL. All substances used in the making up of media were obtained from Difco. The HATF nitrocellulose filters were obtained from Millipore, the nick translation kit, the [^{32}P]α-dCTP, and the

restriction enzymes from Amersham. Lysozyme and salmon sperm DNA were from Sigma; X-ray films, developer and fix were from Kodak. The polynucleotide kinase was from BRL and the [^{32}P]γ-dATP was from Amersham. Otherwise the chemicals used were of the highest possible purity available from Sigma, Aldrich and BDH.

4.2.03 Primary strand synthesis.

(i) Treatment of A+ RNA.

RNA was made up in water and 6μg was used in each 40μl reaction. To remove secondary structure the RNA was heated to 68°C for 5 minutes before adding to the pre-warmed reaction mixture.

(ii) Reaction.

The following reaction mixture was set up in a siliconized eppendorf tube on ice: 1 x RT buffer (50mM Tris-HCl, pH 8.3 at 43°C; 10mM $MgCl_2$; 50mM DTT), 1.25mM dGTP, 1.25mM dTTP, 1.25mM dATP, 0.50mM dCTP, 100μg/ml oligo(dT_{12-18}), 4mM sodium pyrophosphate, 0.43U/μl RNasin, 150μg/ml poly A+ RNA and 1μl of [^{32}P]dCTP. This mixture was mixed and warmed to 43°C, prior to addition of the enzyme, reverse transcriptase at a reaction concentration of 3U/μl.

The reaction was incubated at 43°C for 30 minutes, and then placed on ice. 2µl of 0.50M EDTA was added to stop the reaction.

(iii) Measurement of incorporation.

To calculate the percentage incorporation of label, and hence the amount of cDNA made, the total counts per µl, the background counts per µl and the incorporated counts per µl were measured. The total counts were measured by spotting 1µl of the reaction mix onto a GF/C filter, drying and counting in a toluene based scintillant. The background counts were measured by taking 1µl from the reaction at time zero and transferring it to 500µl of salmon sperm DNA (100µg/ml in 20mM EDTA) on ice in a scintillation vial. The insert was filled with ice cold 10% TCA, mixed and kept on ice for 15 minutes. The TCA suspension was then filtered through a GF/C disc and washed with 6 volumes of ice cold 10% TCA and a final wash of 95% ethanol. The disc was dried and put with scintillant and counted in a Packard scintillation counter. To calculate the incorporated counts 1µl of the reaction volume was taken at the end of the reaction time of 30 minutes and treated as described for the background counts. The amount of cDNA made was calculated by estimating the percentage incorporation by comparing total and incorporated counts, and using this to estimate the total quantity of cDNA, given the concentration of dCTP in the reaction mix.

(iv) Purifying the cDNA.

The cDNA was extracted with 40µl of phenol/chloroform/isoamyl-alcohol (25:24:1) the phenol prepared as described in Maniatis *et al.*, (1982). The aqueous phase was transferred to a fresh siliconized eppendorf tube and the organic phase re-extracted with 40µl of TE buffer (10mM Tris-HCl, pH 8.0; 1mM EDTA, pH 8.0). The aqueous phases were combined and 80µl of 4M ammonium acetate and 320 µl of absolute ethanol were added. The DNA was allowed to precipitate in a dry ice/ethanol bath for 15 minutes, and then warmed to room temperature with gentle shaking. The DNA was pelleted at top speed in a microfuge for 10 minutes, washed with cold (-20°C) 95% ethanol and re-pelleted. The pellet was re-dissolved in TE buffer, pH 7.4 and re-precipitated as described above. The final pellet was vacuum dried and dissolved in TE, pH 7.4, to give a concentration of 100ng of DNA/RNA hybrid per µl. The pellet was stored in the freezer at -80°C if necessary at this stage.

4.2.04 Secondary strand synthesis.

The reactions were carried out in volumes of 100µl. In a siliconized eppendorf tube on ice the following reaction was set up: 1 x SS buffer (20mM Tris-HCl pH 7.5; 5mM $MgCl_2$; 10mM $(NH_4)_2SO_4$; 100mM KCl), 50µg/ml BSA and 40µM of each dNTP. This mixture was mixed and the following added: 1µg of DNA/RNA hybrid, 9U/ml RNase H, 230U/ml DNA polymerase 1 and 1µl of [^{32}P] dCTP (optional). The reaction was mixed and incubated at

12°C for 60 minutes and then 22°C for 60 minutes. The reaction was stopped by adding 5µl of 0.5M EDTA. The incorporation could be measured as described in section 4.2.03 (iii). The double stranded cDNA was then cleaned up in the same way as described in section 4.2.03 (iv), with appropriately increased amounts. The final pellet was dissolved in 20µl of TE, pH 8.0.

4.2.05 Size selection of cDNA.

A 1% w/v agarose gel in TBE buffer (0.089M Tris-borate; 0.089M boric acid; 0.002M EDTA) was prepared and the cDNA was run against digested λ-DNA [prepared as described in section 4.2.13 (ii)] as a molecular weight marker. The gel was run in TBE containing ethidium bromide at 30mA until the bromophenol blue dye in the marker was about a third of the way down the gel. The gel was viewed on a UV light box and an incision made in the gel at the minimum size of interest of the cDNA. A piece of DEAE nitrocellulose NA45 paper (Schliecher and Schull) was then inserted into the cut to collect the cDNA. The paper was prepared by washing in 2.5M NaCl for at least 2 hours, rinsing in TE pH 8.0, and storing in 2mM EDTA at 4°C. The DNA was extracted from the paper by placing the trimmed disc into 300µl of 1.5M NaCl in TE pH 8.0 and shaking at 37°C overnight, or at 65°C for 1 hour. The presence or absence of the DNA on the paper was easily ascertained by viewing in UV. The paper was removed and the cDNA precipitated by adding 600µl of absolute ethanol, and keeping in a dry ice/ethanol

bath for 15 minutes. The pellet was collected at top speed in a microfuge, washed in 95% ethanol and vacuum dried. The final pellet was dissolved in 20µl of water.

4.2.06 Optimization of the tailing reaction.

It was decided to clone the cDNA into the *Pst*1 site of pBR322 by G-C tailing. Commercially dG-tailed plasmid was obtained from BRL, purporting to possess dG tails of approximately 15 residues. Prior to tailing all the cDNA a pilot tailing reaction was performed with a sample of cDNA to determine the optimum tailing time. The following reaction was set up in a total volume of 20µl: 1 x tailing buffer (200mM K cacodylate, pH 6.9; 1mM $CoCl_2$), cDNA (say 5µl), 5mg/ml BSA and 10µM dCTP. These reagents were mixed and pre-warmed to 37°C, prior to addition of the enzyme, terminal transferase, at a concentration of 0.75U/µl. The reaction was incubated at 37°C for 20 minutes and samples were taken at regular intervals, e.g. 0, 5, 10, 15 and 20 minutes. Of these samples, 1µl was TCA precipitated as described in section 4.2.03 (iii) and 2µl annealed to tailed pBR322 and used to transform competent cells which were then plated out, as described in sections 4.2.07 and 4.2.08. This gave an empirical determination of the optimum tailing time. The total counts were calculated by drying 1µl of the reaction mixture onto a GF/C disc and counting as described in section 4.2.03 (iii).

4.2.07 Optimization of the annealing reaction.

The 2µl samples of cDNA obtained from the tailing experiment were annealed as described below to a constant 15ng per reaction volume of tailed plasmid. In order to ascertain the optimal ratio of cDNA to plasmid the following experiment was performed. A variety of concentrations of tailed cDNA were annealed to 15ng of tailed plasmid in reaction volumes of 30µl. The amounts of cDNA ranged from 1µl of 1/10 concentration to 5µl of original concentration. Each reaction contained 15ng of tailed pBR322, 1 x anneal buffer (5mM Tris-HCl, pH 7.5; 100µM EDTA; 75mM NaCl) and cDNA. The reactions were placed in a water bath at 65°C for 10 minutes. After this the temperature of the water bath was adjusted to 58°C and allowed to cool gradually to that temperature for 90 minutes. The tubes were then placed in a small beaker of water at 58°C on the bench and allowed to cool to room temperature. The annealings could either be frozen at this stage or used for transformation.

4.2.08 Preparation and use of competent cells.

The method used to prepare the competent cells was that described by Hanahan (1985) entitled the 'frozen storage method, protocol 3'. This method was chosen as a result of a survey of the most efficient methods of transformation described in Appendix D. A 10ml overnight culture of HB101

cells in L-broth (1% bacto tryptone; 0.5% bacto yeast extract; 1% bacto agar) was set up by inoculating the broth with a scrape from a frozen stock (storage of stocks is described in Appendix C). The culture was incubated with moderate shaking overnight at 37°C. 1ml of this '10ml overnight' was used to inoculate 100ml of warmed L-broth. This culture was incubated at 37°C with moderate shaking until the cells reached a density of 4-7 x 10^7 cells/ml (characterization of the growth of HB101 is described in Appendix C). The culture was collected in polypropylene Sorval 50ml tubes and centrifuged in the Sorval 8 x 50ml rotor at 750-1000*g*. The pellets were drained and resuspended in 1/3 of the original culture volume of RF1 (100mM KCl; 50mM $MgCl_2.4H_2O$; 30mM potassium acetate; 10mM $CaCl_2.2H_2O$; 15% w/v glycerol; to pH 5.8 with 0.2M acetic acid, filter sterilized through a 0.22µm membrane). The cells were incubated on ice for 2 hours, and pelleted as before. The pellet was resuspended in 1/12.5 of the original volume of RF2 (10mM MOPS, pH 6.8; 10mM KCl; 75mM $CaCl_2.2H_2O$; 15% w/v glycerol, to pH 6.8 with NaOH, filter sterilized through a 0.22µm membrane). The cells were incubated on ice for 15 minutes and then aliquoted into eppendorf tubes and flash frozen in a dry ice/ethanol bath before being placed at -80°C.

When required for use the cells were removed from the freezer and allowed to thaw at room temperature until the cell suspension was liquid. The suspension was aliquoted into 200µl amounts (if larger amounts had been used for storage) and

placed on ice. The DNA was added to the tubes in a volume that was less than 20µl, and swirled gently. The tubes were incubated on ice for 10-60 minutes, and the cells were then heat shocked by incubating at 42°C for 90 seconds and then returned immediately to ice for 2 minutes. 800µl of L-broth with 20mM glucose was added and the cells incubated at 37°C for an hour with moderate agitation. Known volumes of the cell dilution were then plated onto tetracycline agar plates.

4.2.09 Plating and replica plating.

Agar plates were prepared by autoclaving L agar (1% bacto tryptone; 0.5% bacto yeast extract; 1.5% bacto agar; 1% NaCl) and allowing it to cool to 45°C. Antibiotics were added from stocks (prepared as in Appendix C) to 50µg/ml ampicillin or 12.5µg/ml tetracycline, when the agar was cool. It was then poured into pre-sterilized agar plates from Sterilin, allowed to set and dried inverted for 10-15 minutes in a sterile cabinet. Known volumes of cell suspension were aliquoted into the centre of the plate and spread with a glass rod, sterilized alternately in ethanol and flame. The plates were incubated at 37°C in an inverted position in the dark.

Colonies so produced were transferred using sterile toothpicks to known positions on tetracycline plates by using a template. The tetracycline resistant colonies were then transferred to the same positions on ampicillin plates to test for ampicillin susceptibility.

4.2.10 Tailing, annealing and cloning the cDNA.

Once the optimal tailing time had been determined it was possible to tail the rest of the cDNA. This was performed as described in section 4.2.06 but scaled up to encompass the total cDNA present. The annealing of the optimally tailed cDNA was performed at the optimum cDNA:plasmid ratio as determined in section 4.2.07. The annealings were transformed into competent cells prepared by the method of Hanahan (1985) 'frozen storage, protocol 3', and plated onto tetracycline as described above in section 4.2.09. Those colonies found to be resistant to tetracycline were replica plated onto ampicillin as described. Those clones found to be tetracycline resistant and ampicillin susceptible were identified as recombinants.

4.2.11 Storage of cDNA clones.

Clones were stored on tetracycline L-agar plates at 4°C. The cells were transferred to fresh plates once every two weeks or so. Ripening specific clones were stored at -80°C in 20% w/v glycerol in L-broth.

4.2.12 Colony Hybridization.

The method used was taken from Hanahan and Meselson (1983).

(i) Preparation of filters.

Colonies were transferred to nitrocellulose filters lying on fresh tetracycline agar plates using sterilized toothpicks. The filters were marked with a grid that corresponded with the template used to define the positions of the colonies on their original plates. The filters were grown for about 5 hours at 37°C until the colonies were visible.

The filters were treated to lyse the cells and denature their DNA. Pads of Whatman 3MM paper was soaked in the relevant solutions and the filters laid gently on top of the pad. In this way the filters were soaked in 0.50M NaOH for 10 minutes, blotted dry, and soaked in fresh 0.05M NaOH for 10 minutes. They were then neutralized by soaking in 1M Tris-HCl pH 8.0 for 10 minutes, followed by 1M Tris-HCl pH 8.0/1.50M NaCl for 10 minutes. The filters were then briefly air dried and baked in a vacuum oven at 70°C for 2 hours.

(ii) Preparation of the cDNA probe.

cDNA was prepared as described in sections 4.2.03 and 4.2.04. 1.0µg of green fruit cDNA and 1.0µg of red fruit cDNA were subjected to the nick translation procedure, which was that recommended by Amersham. To 20µl of cDNA in sterile pure water were added the following, 20µl of buffer/nucleotide solution, 4µl of [^{32}P]α-dCTP, 10µl of enzyme solution (DNase 1 and DNA polymerase 1) and 46µl of water to a final volume of 100µl. Two 2µl samples were taken to estimate the total and background counts as in section 4.2.03 (iii), and at the end

of the reaction a 2µl sample was taken to estimate the incorporated counts. The reaction was incubated at 15°C for 1 hour. 40µl of 0.50M EDTA was added to halt the reaction. The labelled DNA was ethanol precipitated as described in section 4.2.03 (iv), and the final pellet was resuspended in 300µl of TE pH 8.0 and stored briefly at 4°C. Of the 300µl only 100µl was used in the hybridization reaction.

The 100µl aliquot was treated with 10µl of 1.0M NaOH for 2 minutes at room temperature to denature the probe DNA, followed by 10µl of 1.0M Tris-HCl pH 8.0 and 10µl of 1.0M HCl. The denatured neutralized probe was then added to the hybridization solution and the pre-hybridized filters.

(iii) Pre-hybridization of the filters.

The filters were pre-hybridized to block non-specific sites. They were incubated in pre-hybridization solution for 2 hours, with moderate shaking at 42°C. The pre-hybridization solution contained 50% deionized formamide, 5 x Denhardt's solution, 5 x SSPE buffer, 1% glycine and 50µg/ml salmon sperm DNA. Formamide was prepared by stirring with several changes of a mixed bed resin (Dowex) and filtering into covered bottles to be stored at -20°C. 50 x Denhardt's solution was made by dissolving 5g of Ficoll, 5g of polyvinylpyrrolidone and 5g of BSA in 500ml of water. The solution was filtered and stored in aliquots at -20°C. 20 x SSPE buffer was made by dissolving 17.4g of NaCl, 2.76g of $NaH_2PO_4.H_2O$ and 0.74g of EDTA in a

final volume of 100ml of water. The pH was adjusted to 7.4 with NaOH. The solution was sterilized by autoclaving and stored at -20°C in aliquots.

2.5ml of pre-hybridization solution was used per filter, and each filter was sealed into a separate polythene bag.

(iv) Hybridization.

The pre-hybridization solution was squeezed out of the bags and hybridization solution was added. Hybridization solution consisted of 62.5% deionized formamide, 5 x SSPE, 1.25 x Denhardt's solution, 0.2% SDS and 0.25mg/ml salmon sperm DNA, the formamide, SSPE and Denhardt's solution prepared as described in section 4.2.12 (iii). The probe was treated as described in section 4.2.12 (ii) and added to the hybridization solution. The filters were incubated in this solution for 12 hours with moderate shaking at 42°C.

(v) Washing the filters.

The (radioactive) hybridization solution was carefully transferred to sterile bottles and stored at -20°C in case it was needed again. The filters were washed at room temperature with vigorous shaking in a high salt (high stringency) wash (2 x SSC buffer; 0.1% SDS). SSC buffer consisted of 0.15M NaCl/15mM sodium citrate, to pH 7.0 with HCl and autoclaved to sterilize. 4 washes were performed, for 5 minutes per wash. The washed filters were carefully blotted dry and mounted on

Whatman 3MM. They were marked with radioactive ink and a nick and wrapped in cling film. They were then placed under an X-ray film in an autoradiograph cassette and left at -70°C to develop.

(vi) Developing the autoradiographs.

The autoradiographs were developed as described in section 3.2.15 (iii), that is they had 5 minutes in developer and 3 minutes in fixative, were rinsed in distilled water and air dried.

4.2.13 Minipreparations of ripening specific clones.

(i) Isolation of plasmid DNA.

Colonies were chosen that demonstrated stronger hybridization with the red probe than with the green. These colonies were thought likely to contain inserts representing red fruit specific RNA. Of the 500 recombinants screened 18 were judged to be ripening specific on the basis of their relative hybridization to the red probe. These cell lines were grown up and minipreparations were made of them to enable the inserts to be looked at more closely. The method of making minipreps was that recommended by Dr C.M.Lazarus of Bristol University. 10ml of L-broth containing 12.5µg/ml tetracycline was inoculated with each of the clones of interest, by taking a scrape with a sterile toothpick. The 10ml overnight was grown at 37°C, shaking, overnight. The cultures were centrifuged at

4,000 r.p.m. in a Sorval 8x50ml head, and the pellet drained and resuspended in 50µl of sucrose buffer (25% sucrose; 50mM Tris-HCl pH 8.0). 25µl of freshly made 20mg/ml lysozyme/10mM Tris-HCl, pH 8.0 was added, the tubes flicked to mix the enzyme, and then left on ice for 5 minutes. 63µl of 0.2M EDTA pH 8.0 was added and the tubes left for a further 5 minutes on ice. 163µl of Triton lysis buffer (0.1% Triton x 100; 50mM Tris-HCl pH 8.0; 65mM EDTA) was added and the cells left for 15 minutes on ice. The cell suspension was then frozen in dry ice, allowed to thaw, and centrifuged at top speed in a microfuge. The supernatant was drawn off from the sludge constituting the lysed cells and transferred to a fresh tube. The DNA solution was then phenol extracted as described for cDNA in section 4.2.03 (iv) and ethanol precipitated as also described in that section.

(ii) Digestion of the recombinant plasmid.

Digestions were set up in siliconized sterile eppendorf tubes. The DNA was dissolved in water so that no buffer constituents would interfere with the digestion. In general digestions were performed in final volumes of 10µl, and contained the DNA (in water), 1µl of 10 x core buffer (500µg/ml BSA; 500mM NaCl; 500mM Tris-HCl pH 8.0; 100mM $MgCl_2$; 10mM DTT) and a suitable quantity of enzyme (1U of enzyme is defined as sufficient to digest 1µg of DNA in 1 hour), made to the final volume with sterile pure water. The digestions were incubated at 37°C for up to 3 hours. The reactions were stopped by heat denaturing

the enzyme at 65°C or by freezing at -20°C, or by loading straight onto a gel.

(iii) Gel electrophoresis of minipreps.

1.1% w/v agarose gels were made in 1 x TBE buffer (0.089M Tris-borate; 0.089M boric acid; 0.002M EDTA) and the digested miniprep DNA was run alongside a λ-DNA marker, digested with *Hin*dIII and *Eco*R1 (as described in section 4.2.13 (ii)) and both native pBR322 and native undigested miniprep DNA. This was so that the insert size could be accurately gauged, and to check that the insert was actually a result of digestion and not a constant artifact in the DNA. The digested DNA was mixed with 6 x loading buffer (0.25% bromophenol blue, 40% w/v sucrose). The whole sample was generally loaded into the well and the gels were run at 60mA until the dye had reached about a third of the way down the gel. Gels were stained by adding a few drops of 10mg/ml ethidium bromide to the running buffer and leaving for 15 minutes, and destained for about 5 minutes in distilled water. The gels were then viewed on a UV light box and photographed using a polaroid land camera and a polaroid 665 black and white film.

4.2.14 Maxipreparations of ripening specific clones.

Four clones were judged to be of sufficient size to be worth subjecting to a maxiprep procedure. These clones were grown up in a 10ml overnight as described for the miniprep procedure in

section 4.2.13 (i), and a 1ml inoculum used to start 1l cultures grown in 2l flasks with 12.5µg/ml tetracycline. The cultures were grown at 37°C with hard shaking until the OD_{600} of the culture was 0.8, measured against an L-broth blank. 150µg of chloramphenicol, and 4g of glucose were added and the cultures allowed to shake overnight, for an optimum of 16 to 18 hours. The cultures were centrifuged at 5,000 r.p.m. for 10 minutes in a pre-chilled Sorval 300ml angle rotor at 4°C. The pellet of cells was scraped out of the 300ml bucket and transferred to a Sorval 50ml tube. The 300ml tube was rinsed with 4ml of 10% sucrose/2mM $MgCl_2$ and this rinse was transferred to the 50ml tube. The cell suspension was vortexed until the cells were uniformly resuspended leaving no lumps. The cells were then chilled on ice. 0.7ml of freshly made up 10mg/ml lysozyme/10mM Tris-HCl pH 8.35 was added and mixed by shaking (not whirlymixing). The tubes were left for 5 minutes on ice, 0.5ml of 0.5M EDTA was added and then they were left a further 5 minutes. 6ml of Triton buffer was added (0.5% Triton x 100; 62.5mM EDTA; 50mM Tris-HCl pH 8.35), mixed by inverting, and the cultures left on ice for 20 minutes and mixed by inverting every 5 minutes. The lysed cell debris was pelleted by centrifuging in a Sorval 8x50ml rotor at top speed for 10 minutes at 4°C. The supernatant was decanted into pre-weighed universal bottles and for each gram of supernatant 1g of CsCl was added and 0.1ml of 10mg/ml ethidium bromide. This solution was shaken to dissolve the salt, and left to stand for 1 hour at 4°C. It was centrifuged briefly (5 minutes) in a

bench centrifuge to pellet solid material, and the liquid phase collected and filtered if necessary. The liquid phase was put into a Beckman quick seal 5ml tube and centrifuged at 50,000 r.p.m. in a Beckman Vti65 rotor, at 10°C, for 16 hours or more. The lower band was collected by cutting the top off of the tube and puncturing the side of the tube with a syringe as described in section 3.2.04 (ii). CsCl was removed from the plasmid band by centrifuging through a 0.22μm membrane using an Amicon Concentricon, and repeatedly rinsing with sterile TE pH 8.0. Ethidium bromide was removed from the sample by extracting several times with isoamylalcohol. The final aqueous phase was ethanol precipitated by adding 0.1 volumes of 3M sodium acetate and 6 volumes of ethanol, and kept overnight at -20°C. The DNA was centrifuged at 10,000g for 30 minutes at 4°C. The pellet was vacuum dried, resuspended in pure sterile water, and stored at -80°C.

4.2.15 Extraction of insert.

Maxiprep DNA was digested with *Pst*1 as described in section 4.2.13 (ii). The digest was run on a gel as described in section 4.2.13 (iii). Inserts were collected on prepared NA45 paper as previously described for cDNA in section 4.2.05. The DNA on the paper was eluted as described in that section and the ethidium bromide removed as described above in section 4.2.14.

4.2.16 Hybridization of inserts against green and red fruit RNA.

Total fruit RNA was extracted using the non frozen phenol/choloroform method described in section 3.2.06. The RNA was selected by oligo(dT) cellulose chromatography as in section 3.2.13 and subjected to denaturing gel electrophoresis as described in section 3.2.11. The gel consisted of two sets of three tracks, red A+ RNA, green A+ RNA and TMV RNA. This was so that two identical filters could be made, each containing red and green A+ RNAs and a control track of TMV RNA.

(i) Preparation of the RNA filters.

A shallow tray was set up containing a low level of buffer (3M NaCl; 0.3M sodium citrate). Into this buffer was placed a stack of sterile Whatman 3MM paper, so that the buffer soaked through. The gel was placed on this stack and a piece of nitrocellulose paper placed on the gel. A further stack of Whatman 3MM was added, followed by a wad of dry material (disposable nappies) and a heavy weight. The RNA was left to transfer to the nitrocellulose by capillary action overnight at room temperature. Care was taken to ensure that the dry material above the gel did not come into contact with the wet material below the gel, so the buffer could not bypass the gel. All buffers and materials coming into contact with the

RNA were sterile. The method is that recommended by Hatfield Polytechnic (Nucleic Acids Workshop).

(ii) Preparation of the probes by end-labelling.

The following reaction was set up in a siliconized eppendorf on ice: 10µl of insert DNA, 10µl of [^{32}P]γ-dATP (5µCi), 5µl of 10 x kinase buffer (0.5M Tris-HCl, pH 7.6, 0.1M $MgCl_2$, 50mM DTT, 1mM spermidine, 1mM EDTA), 1µl of T4 polynucleotide kinase, to 50µl with pure sterile water. The reaction was incubated at 37°C for 30 minutes, and stopped by adding ammonium acetate to 0.3M. The labelled DNA was ethanol precipitated, and finally resuspended in 50µl of pure water. The specific activity of the probes was calculated by taking a 2µl sample at time zero and at the end of the reaction and allowing them to be absorbed onto a DE-81 filter. The filters were subjected to 6 washes of 5 minutes each in 0.5M Na_2HPO_4, 2 washes of 1 minute each in water and 2 washes of 1 minute each in 95% ethanol. The discs were then dried and put with 3ml of optiphase scintillant, and counted in a Packard scintillation counter. The total counts were estimated by counting an unwashed disc. When the filters were ready for the hybridization step the probes were denatured by heating to 100°C for 15 minutes and added into the hybridization buffer.

(iii) Pre-hybridization of the filters.

The method was taken from Thomas (1980). The nitrocellulose filters were baked in a vacuum oven at 80°C for 2 hours. They

were then incubated in pre-hybridization buffer (50% deionized formamide; 5 x SSC; 50mM sodium phosphate pH 6.5; 250µg/ml denatured salmon sperm DNA; 0.02% BSA: 0.02% Ficoll; 0.02% polyvinylpyrrolidone) for 15 hours at 42°C with moderate shaking. The formamide and SSC buffer were prepared as described in section 4.2.12 (iii).

(iv) Hybridization of the filters.

The denatured probes were added to hybridization buffer (4 parts pre-hybridization buffer and 1 part 50% dextran sulphate) which in turn was added to the filters. The filters were hybridized for 20 hours at 42°C, with moderate shaking.

(v) Washing the filters.

The filters were subjected to a low stringency (high salt) wash. The high stringency wash recommended by Thomas (1980) was left out. The filters were washed 4 times, each of 5 minutes duration in 2 x SSC/0.1% SDS. The filters were then air-dried and wrapped in cling film, before being placed under an X-ray film and left at -70°C in an autoradiograph cassette.

(vi) Developing the autoradiograph.

The autoradiograph was developed as previously described in section 3.2.15 (iii). The film was left to expose for 2 weeks due to the low specific activity of the probes.

4.3.00 Results and Discussion.

Previous work (chapters 2 and 3) has shown dramatic alterations in the pigmentation, ultrastructure, protein population, and mRNA population of pepper fruit as they develop from unripe to ripe. My objectives, however, were not only to demonstrate that changes do occur, but to probe how and why they occur. These investigations were to be conducted using a ripe fruit derived cDNA library. The rationale was to create double stranded cDNA from a ripe fruit mRNA population, and use the cDNA inserted into a plasmid, to transform competent *E.coli* cells. Transformants and recombinants were to be selected by methods suitable for the system used, and the recombinant cDNA library screened in duplicate with either red or green fruit derived labelled cDNA. The screening procedure should reveal clones that hybridized preferentially to the red fruit derived probe, and so allow selection of ripe fruit associated inserts.

The system finally adopted for the selection of pepper fruit cDNAs was vector pBR322 and host *E.coli* strain HB101. Homopolymer tailed cDNA was annealed into tailed pBR322 at the *Pst*I site in the ampicillin resistance gene of pBR322. Tetracycline resistance was initially used to select transformants, and ampicillin sensitivity used to select for recombinants. Recombinant colonies were grown on

nitrocellulose filters, the cells lysed, and the DNA denatured and fixed. Duplicate filters were screened with red or green fruit derived labelled cDNA, and those colonies hybridizing more strongly to the red fruit specific cDNA were interpreted as representing ripe fruit associated sequences. Similar strategies have been used in the establishment of a ripening associated avocado cDNA library, in experiments designed to study the expression of the cellulase gene (Christoffersen *et al.*, 1984). A tomato ripening associated library designed to study the expression of polygalacturonase was also established in a similar way to the methods described in this work (Slater *et al.*, 1985); also the establishment of a library to study non-specific ripening-related sequences (Mansson *et al.*, 1985). The identification of specific cDNAs such as those coding for cellulase and polygalacturonase was carried out using electrophoretic comparison of the hybrid release *in vitro* translated product with the purified native protein, and immunoprecipitation of the clone *in vitro* translation product with previously raised antisera.

4.3.01 cDNA synthesis.

The method of making cDNA from intact, biologically active mRNA was that recommended by Dr J.Beeching, University of Bath. Prior to cDNA synthesis it is possible to enrich for particular RNAs. This could have been done by loading A+ RNA

on a sucrose gradient to fractionate according to size, and translating, *in vitro*, fractions from the gradient. The fractions that gave products of a ripening associated nature could be pooled, and ripening specific messages thereby enriched. The problem with this approach is the quantity of A+ RNA required, which is at least 100 µg. Pepper tissue yields about 10 µg of total RNA per g fresh weight, therefore extractions of 1000 g of fruit tissue would have to be performed to produce sufficient RNA for one sucrose gradient. Enrichment of a particular RNA species is only necessary if the RNA is degraded or the message rare, neither of which applied to this work. The method used to synthesize cDNA was that developed by Gubler and Hoffman (1983) from an earlier method developed by Okayama and Berg (1982). First strand synthesis was primed by addition of a short oligo (dT) primer to the poly A+ RNA, and a complementary strand of DNA was synthesized by avian myeloblastosis virus (AMV) reverse transcriptase. The synthesis of the primary strand was followed by measuring the incorporation of ^{32}P[dCTP] as described in section 4.2.03 (iii). Synthesis of the second strand was mediated by *E.coli* RNase H, which preferentially degrades or 'nicks' the RNA strand of the DNA-RNA hybrid. DNA polymerase I then synthesizes DNA at the sites of the nicks in a 5' to 3' direction. This method eliminates the need for S1 nuclease digestion of the single stranded hairpin loop, previously used to prime synthesis of the second strand, which resulted

in loss of cDNA. The double stranded cDNA resulted from the RNase H mediated method is blunt at one end, but may have a short 3' overhang at the other:

3'_____ 5'

5'TTTTT_____ 3'

Figure 1 shows a calculation of the quantity of pepper cDNA made using this method. Several attempts were made to synthesize cDNA which were initially unsuccessful. The problems primarily lay with impure or degraded RNA, and much work had to be done to overcome this, and improve RNA quality. Initial attempts at cDNA synthesis were made using a cDNA synthesis kit from P and S Biochemicals Ltd, which also used the RNase H mediated second strand synthesis. A system using pUC8 and *E.coli* strain MC1022 was characterized, but abandoned due to expense and lack of success. I decided to follow Dr J.Beeching's protocol, as it was more detailed than most other methods available, and more economical in terms of materials.

4.3.02 Size selection of cDNA.

Size selection of cDNA is shown in Figure 2. The cDNA was size selected to eliminate molecules too small to be of interest. Short molecules seem to be generated during cDNA synthesis, possibly primed by RNA fragments (Heidecker,

Figure 1. Calculation of [total cDNA] made, using the TCA precipitated samples from the primary strand synthesis reaction.

B = Background counts = 915 c.p.m.
I = Incorporated counts = 7647 c.p.m.
T = Total counts = 722056 c.p.m.

The final proportion of label incorporated is;

$$\frac{(I-B)}{(T-B)} = \frac{(7649-915)}{(722056-915)} = 9.3 \times 10^{-3} = 0.9\% \text{ incorporation}$$

dCTP is at a concentration of 1.25 mM, in a volume of 43 µl;

$$\text{Total moles of dCTP} = \frac{1.25 \times 10^{-3}}{1 \times 10^{6}} \times 43 = 5.37 \times 10^{-8}$$

Of which 0.9% is made into cDNA;

$$0.9\%(5.37 \times 10^{-8}) = 4.77 \times 10^{-10}$$

This represents ¼ of the number of bases, of approximate molecular weight 375, and is for one strand only.

$$\text{Total cDNA} = 4.77 \times 10^{-10} \times 4 \times 375 \times 2 = 1.43 \times 10^{-6} \text{ g}$$
$$= 1.43 \text{ µg (approx)}$$

This represents approximately 20% incorporation.

Figure 2. Size selection of cDNA.

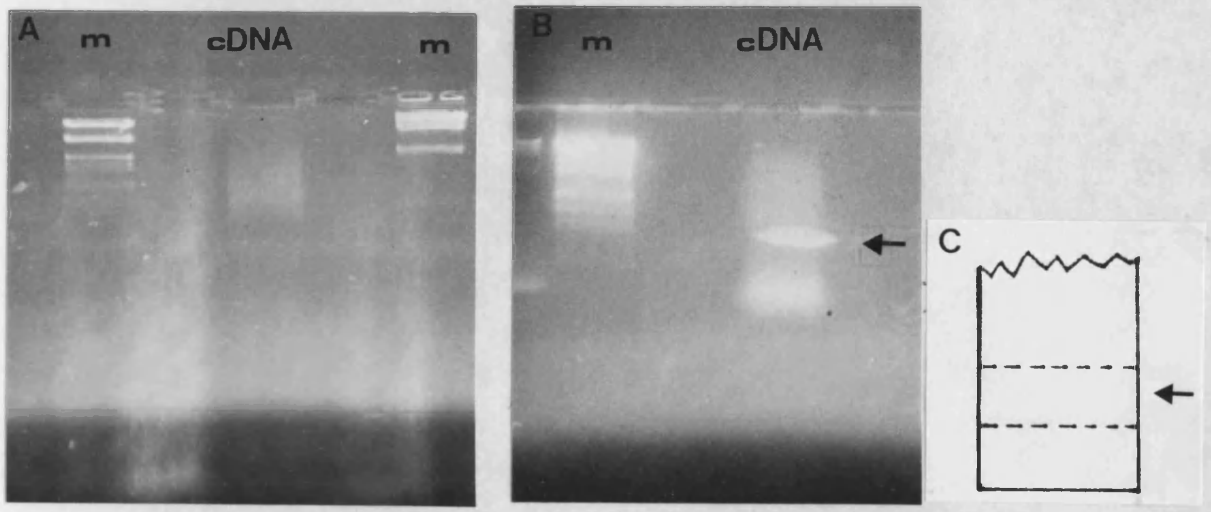


Figure 2. cDNA was run on a 1.1% agarose minigel alongside a marker (m) of *Hin*dIII-*Eco*RI digested λDNA (part a). When the marker sizes became distinguishable the gel was lifted from the tank (with the current turned off), a slit made at the appropriate size in the cDNA track and the treated NA45 paper inserted (part b, arrow). The gel was returned to the tank and electrophoresis continued in the same direction as before. The cDNA was allowed to run into the paper where it collected in a band visible in UV light, diagrammed in part c (arrow). The cDNA was purified from the NA45 paper as described in section 4.2.05.

Figure 3. Empirical optimization of the tailing reaction.

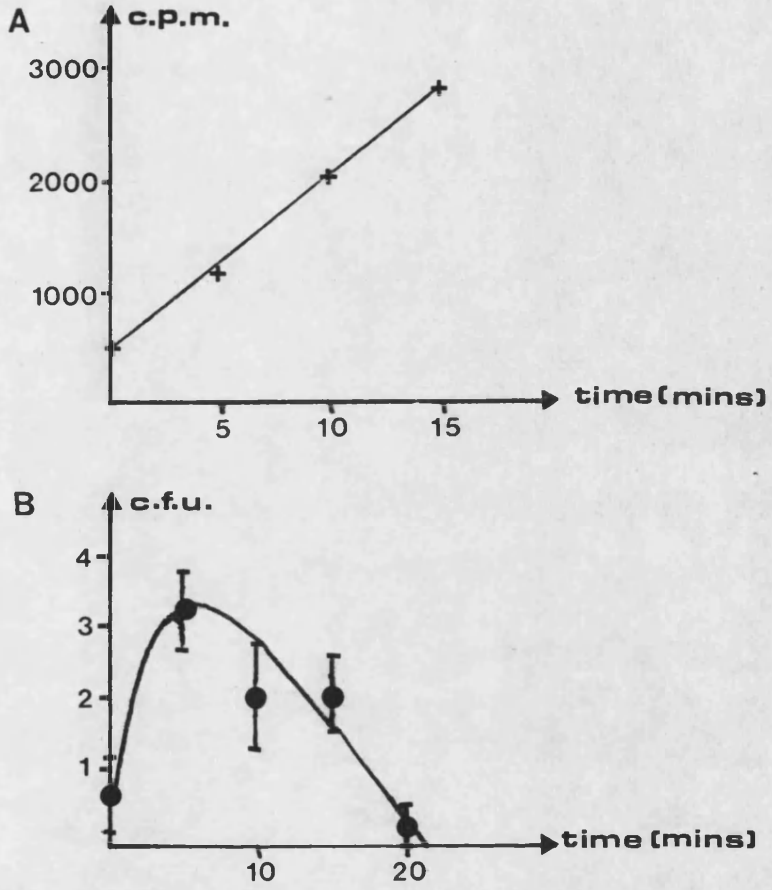


Figure 3. cDNA was tailed as in section 4.2.06, and 2 µl samples taken at regular intervals (e.g. 0, 5, 10, and 15 min) during the tailing reaction. These samples were annealed to a constant 15 ng of dG-tailed plasmid and the annealings used to transform aliquots of competent cells. An aliquot of the transformed cells was plated onto tetracycline agar and the number of tet resistant colonies counted. The time point which yielded the highest number of resistant colonies was judged to be the most suitable, in terms of number of tails added. This time of tailing was used in the scaled up tailing reaction. Part a shows the increase in c.p.m. of 2 µl samples taken at various time-points during the experiment, and part b shows the average number of resistant colonies produced by annealing and transformation of each time-point sample. The most colonies were produced at a tailing time of 5 min. n = 4 for part b.

Figure 4. Empirical optimization of the annealing reaction.

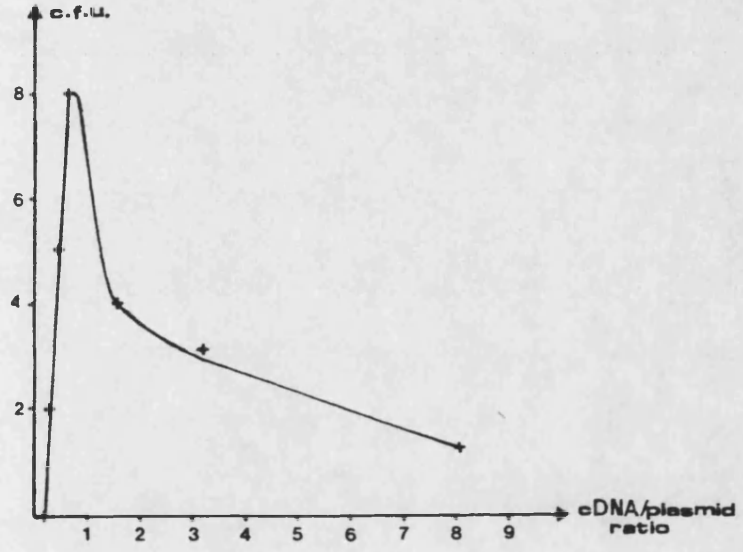


Figure 4. In order to ascertain the optimal ratio of cDNA to dG-tailed plasmid, variable amounts of cDNA were annealed to a constant 15 ng of plasmid. Analysis was by counting the number of colony forming units (c.f.u.) produced from each annealing reaction, after transforming competent cells. The annealing was carried out as described in section 4.2.08. The figure shows the results of one experiment. The optimal ratio was taken to be 0.8:1, approximately 1.1, when the total DNA in the reaction volume of 30 µl is 55 ng.

1983). Radioactive double stranded cDNA was run on a 1% agarose gel alongside a *Hin*dIII-*Eco*RI digested λDNA marker. When the marker sizes became apparent, treated NA45 paper was inserted into the gel to pick up the cDNA of over a minimum size of interest (500 bp). The method is described in section 4.2.05. Overall this method of size selection seemed very simple and successful, although occasionally problems were experienced with the removal of DNA from the paper. A possible problem with this technique is that agarose contamination can prevent ligation of DNA into plasmids (J.Cavell, personal communication). It may have been better to size select the cDNA by a different method, which did not involve agarose, e.g. a glycerol gradient.

4.3.03 Tailing and transformation.

DNA thus sized was treated with butan-1-ol to remove ethidium bromide, precipitated, re-dissolved, and subjected to a tailing reaction as described in section 4.2.06. Figure 3A shows the incorporation of radioactivity into a constant amount of precipitated cDNA as reaction time increases. The enzyme used to tail the cDNA was calf thymus terminal transferase, which adds dNTPs to a free 3' hydroxyl group.

3'CCCCCCC_____ 5'

5'_____ CCCCCCC 3'

The tailed cDNA was then annealed to commercially obtained dG-tailed pBR322. The plasmid had been linearized with *Pst*I and the tails added to the 3' strands at the break. This annealed plasmid-cDNA hybrid was used to transform competent *E.coli* strain HB101. The transformation procedure was analysed as presented in Appendix D. The optimum procedure for our laboratory, using our water and reagents was determined, and used in these experiments. The concentration of DNA in an annealing reaction is adjusted to promote re-circularization of hybrid plasmids rather than the formation of concatemers (Heidecker, 1983). The optimum annealing concentration of DNA in this work was determined empirically by maximizing the number of transformants. Controls of annealed dG-tailed plasmid only, and no DNA were also transformed to assess re-circularization of the tailed plasmid to itself and to monitor the antibiotic sensitivity of the cells. Both these controls routinely gave no transformants. Repair of the annealed plasmid within the cell leads to regeneration of a *Pst*I site on either side of the inserted cDNA (Maniatis *et al.*, 1982). Figure 3B shows the number of colonies obtained from annealing a constant amount of tailed cDNA to 15 ng of tailed plasmid, and transforming the standard aliquot of 200 µl of competent cells. The number of resistant colonies was low, and it was thought this was due to the fairly rigorous size selection of cDNA. In retrospect the numbers obtained were much too low, and possible reasons for this include agarose

contamination of cDNA interfering with the annealing and transformation reactions, or use of a *RecA-* strain of *E.coli*, rather than a *RecA+* strain (Peacock *et al.*, 1981). This is discussed more fully later. Figure 4 shows the next stage of optimization, which involved an empirical analysis of the optimal plasmid:cDNA ratio, again determined by counting transformant colonies. The optimal plasmid:cDNA ratio was found to be approximately 1:1 from this experiment.

4.3.04 Selection of recombinant clones.

Scaled up tailing and annealing reactions were set up using the optimal conditions determined previously, with the rest of the size selected cDNA. Resistant colonies were transferred to numbered positions on a tetracycline plate, using sterile toothpicks. A diagram of the grid used to identify each colony is shown in Figure 5. The positions were duplicated to ampicillin plates, and susceptible colonies were transferred to a master plate. About 500 $tet^{R}amp^{S}$ recombinant colonies were produced, and these constituted the pepper cDNA library used in subsequent manipulations. A criticism of the way the library was obtained is that it was obtained in 'stages' from the experiments designed to optimize the reactions, as well as from the final scaled up tailing, annealing and transformation reaction. This method of acquiring

recombinant clones may produce a library not representative of the cDNA population available. At each stage, certain sequences may be more likely to clone successfully, and by performing several low yielding cloning procedures, it is possible that there will be selection for this limited number of sequences (Dr P.Towner, personal communication). Selection for such sequences would occur in any method of cloning, but inefficient, low yielding cloning procedures would probably emphasize the presence of these sequences in a final library.

4.3.05 Screening the library for ripening related clones.

The strategy used to screen the library was one adapted for selection of abundant messages (Maniatis *et al.*, 1982). Analysis of protein and RNA population changes during ripening revealed several ripening associated products (approximately 40% of the major protein products identified by SDS-PAGE were found to be ripening associated). I was not searching for a particular message, but for a group of fairly abundant messages, and therefore felt that a differential screening strategy was likely to be productive. The method used was that described by Grunstein and Hogness (1975). Colonies were grown on Millipore nitrocellulose HATF filters as recommended by Hanahan and Meselson (1980, 1983) in the presence of tetracycline. Growth was continued overnight and then the cells lysed and the DNA denatured and

Figure 5. Transfer of tetracycline resistant, ampicillin sensitive colonies to a grid position on a master plate.

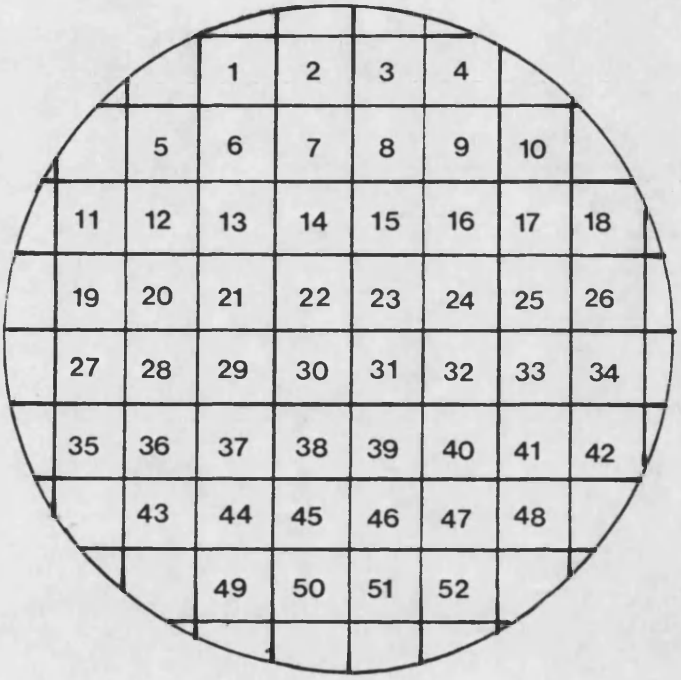


Figure 5. Clones were transferred using sterile toothpicks to a specific grid position. The orientation of the grid was maintained by making a nick at the top right hand 'corner' and a different cut at the top left hand 'corner'. The diagram shows the grid used. Plates were named alphabetically and the colonies numbered 1 to 52. Thus any clone had a letter and numerical designation, e.g. H28, A24.

Figure 6. Calculation of the specific activities of the red and green ds cDNA probes.

cDNA was made as previously described in section 4.2.03, and the quantity of cDNA calculated as in Figure 1. 1 µg each of red and green cDNA was nick translated as described in section 4.2.12 (ii).

For the green cDNA;

B = Background = 12477 c.p.m.
I = Incorporated = 126347 c.p.m.
T = Total = 200204 c.p.m.

$$\text{Percentage incorporation} = \frac{(I-B)}{(T-B)} = \frac{115870}{187727} = 0.617$$

= 61.7% incorporation

Specific Activity;

In 2 µl there are 126347 counts, therefore in 100 µl (1 µg) there are 6417350.

i.e. 6.4×10^6 c.p.m./µg.

For the red cDNA;

B = 2956 c.p.m.
I = 57109 c.p.m.
T = 263999 c.p.m.

$$\text{Percentage incorporation} = \frac{(I-B)}{(T-B)} = \frac{54153}{261043} = 0.207$$

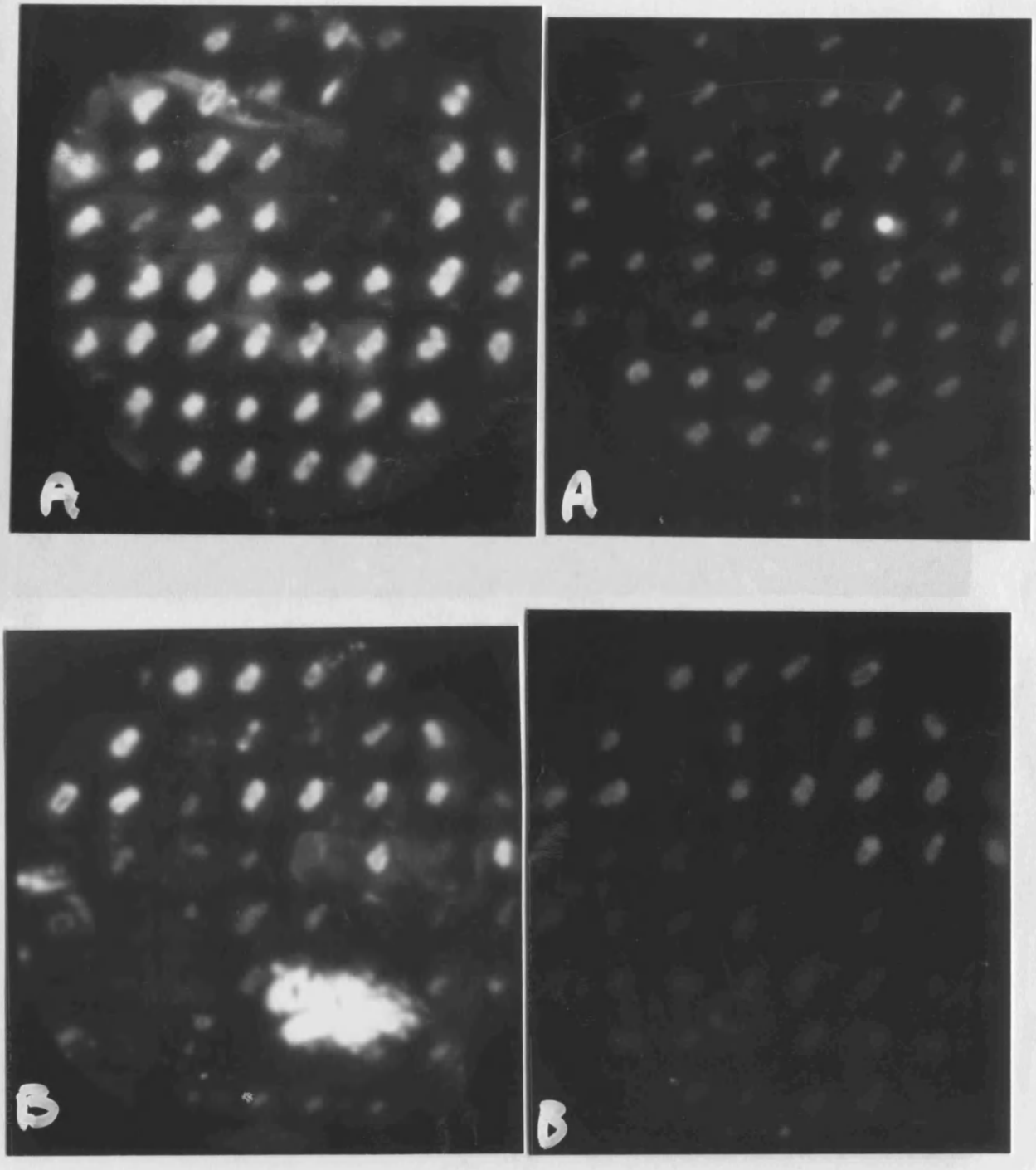
= 21% incorporation

Specific Activity;

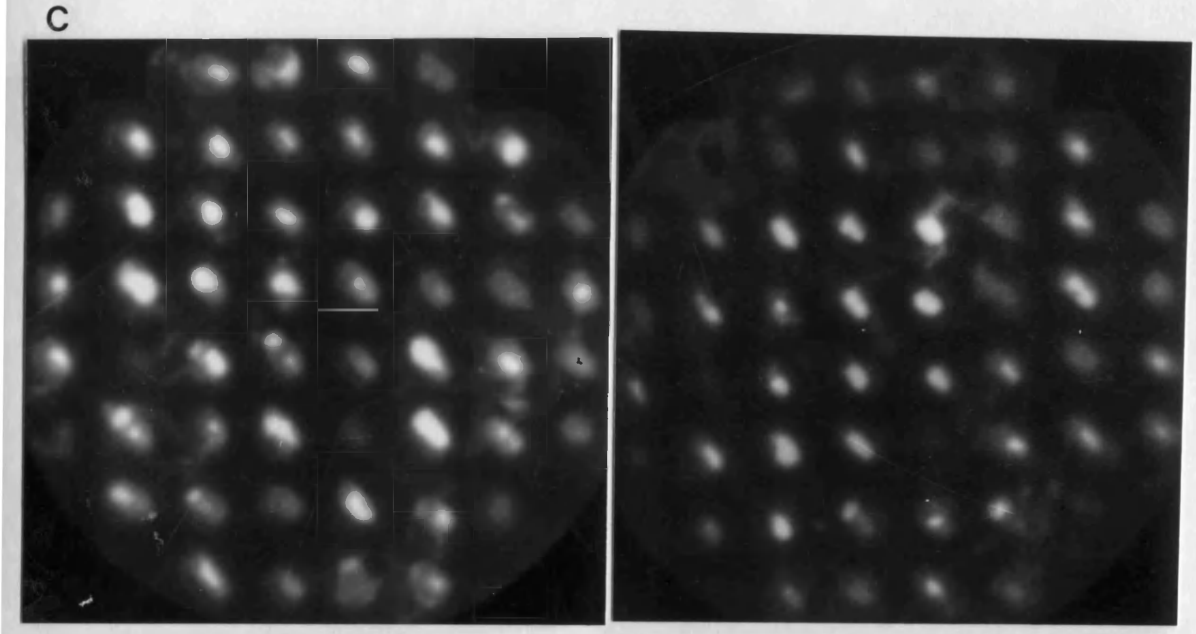
In 2 µl, 57109 c.p.m.
In 100 µl (1 µg), 2855450 c.p.m.

i.e. 2.8×10^6 c.p.m./µg.

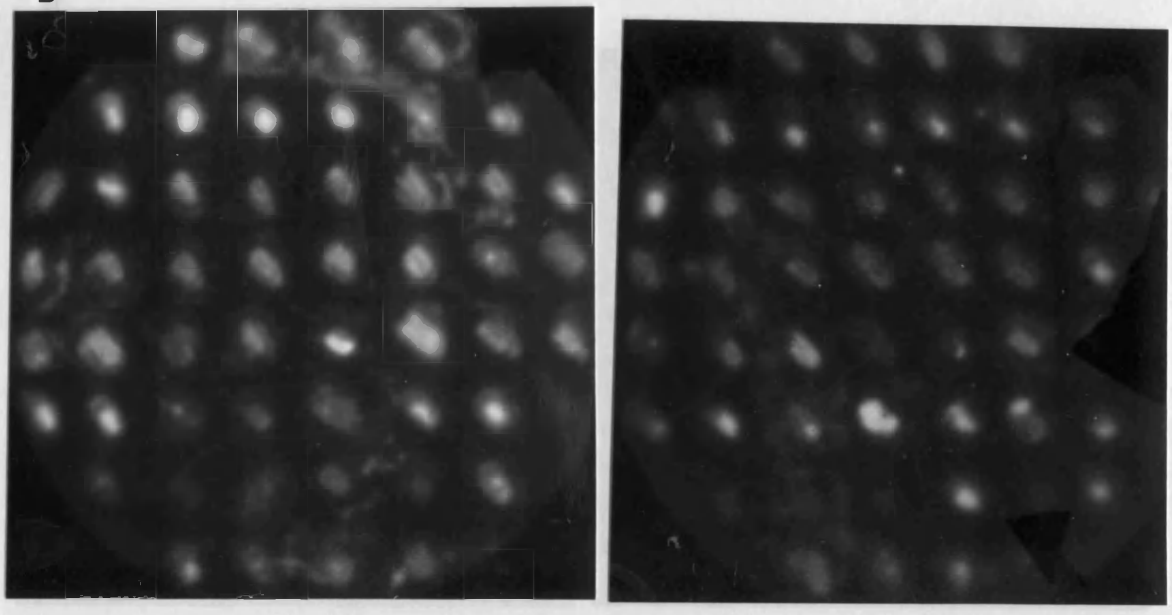
Figure 7. Colony hybridization of ripe fruit cDNA-containing clones with labelled red and green cDNA.

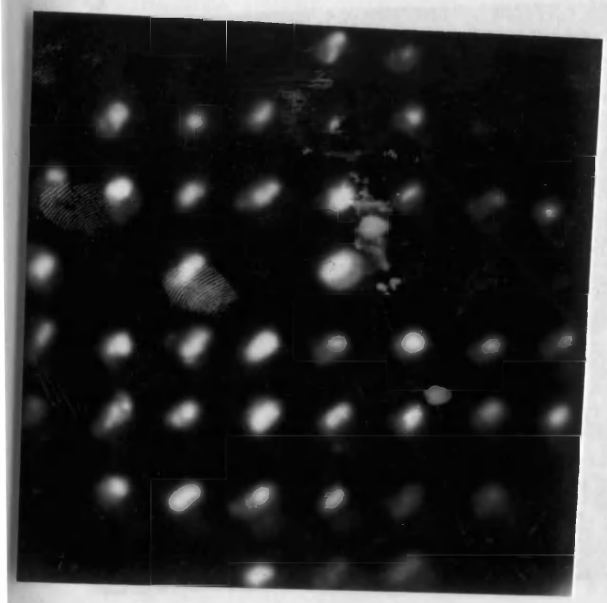


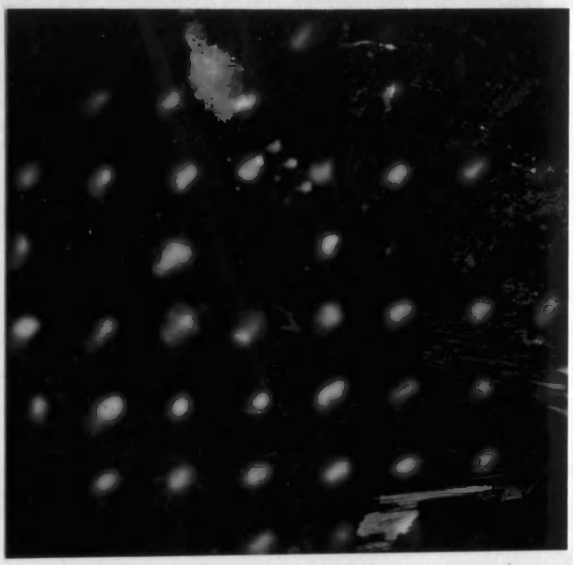
C



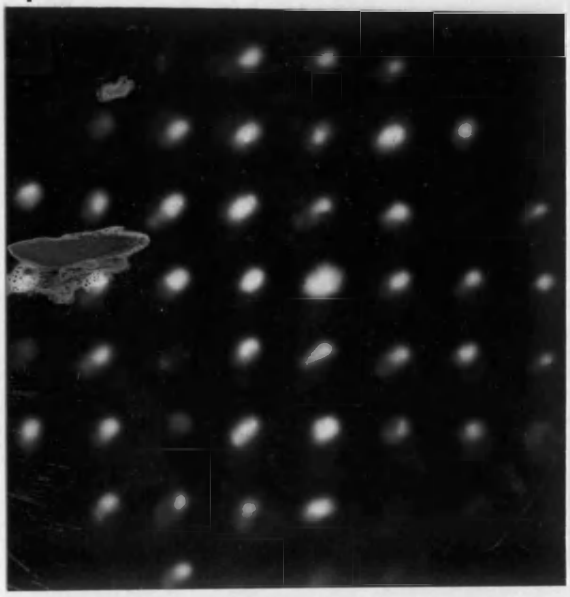
D

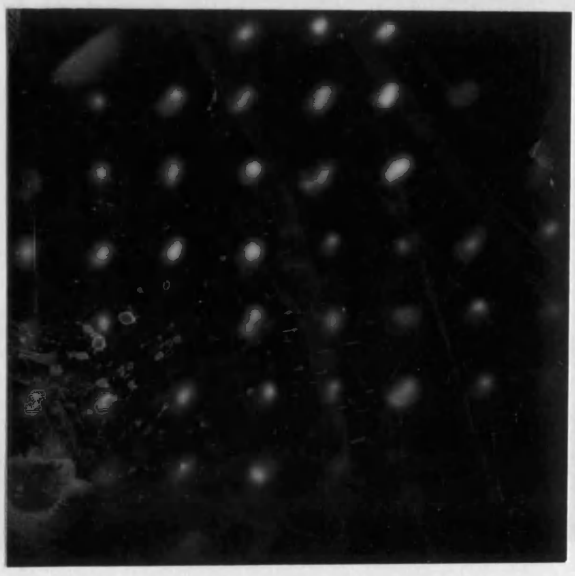




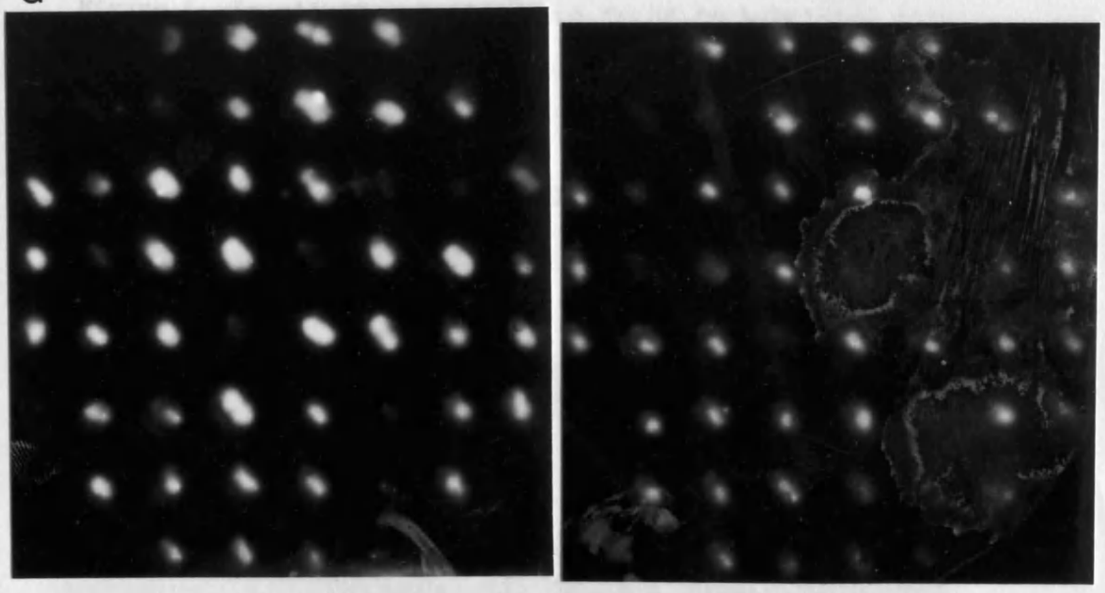


F





G



H

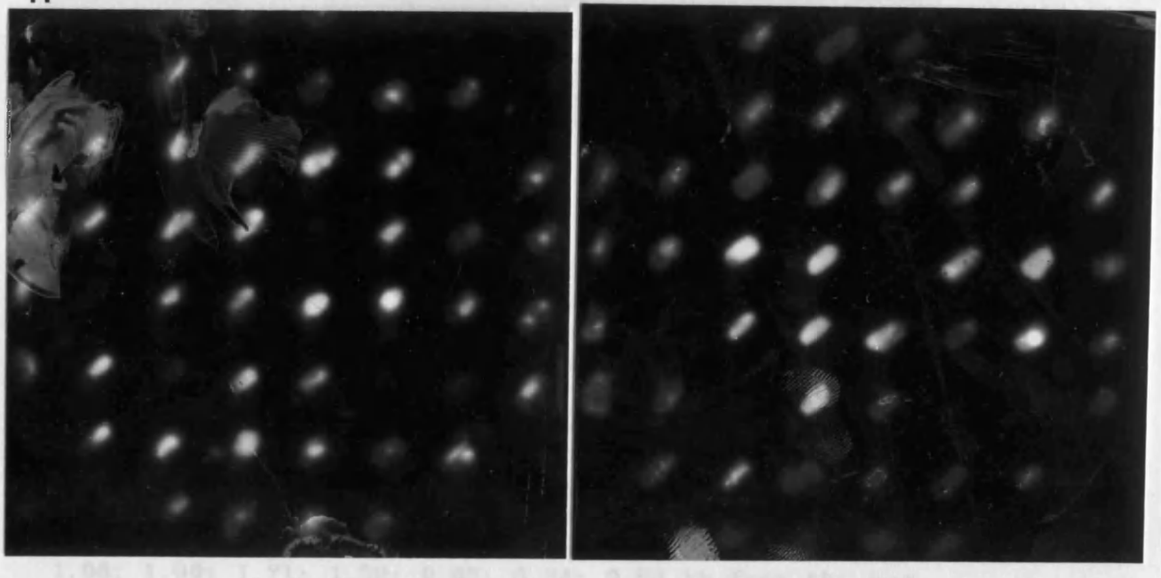


Figure 7. Recombinant colonies were grown on filters as described, and treated to lyse the cells and denature and fix the DNA to the filter (section 4.2.12). Duplicate filters of each plate were then incubated with either red or green labelled cDNA, the excess label washed off and the filter exposed to Kodak X-ray film. Plates were labelled A to H, with R indicating a red probe and G indicating a green. By comparing the red and green hybridization intensities of each colony, it is possible to pick out colonies that hybridize to the red cDNA only, and so possibly contain ripe fruit specific cDNAs. Each colony was identified according to its grid position as outlined in Figure 5. In all 18 colonies were found to hybridize only, or strongly, to the red.

Figure 8. Minipreparations of clones hybridizing to the red cDNA.

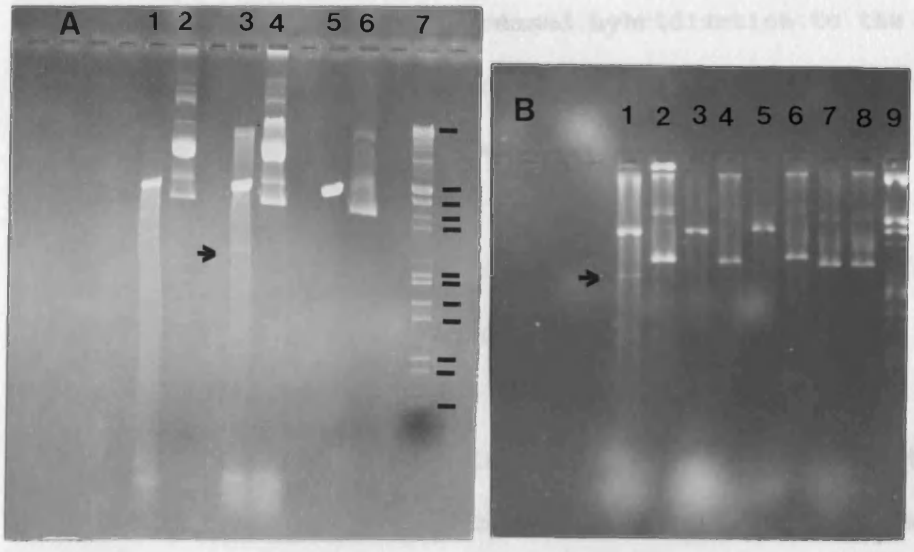


Figure 8. Minipreparations of all red hybridizing clones were made as described in section 4.2.13, digested with *Pst*1 and run on 1.1% agarose gels, alongside a *Hin*dIII-*Eco*RI digested λDNA marker, and both native and linearised pBR322. (A) Lanes are as follows: 1, digested H28; 2, native H28; 3, digested A24, showing an insert at about 2 kb (arrow); 4, native A24; 5, linearised pBR322; 6, native pBR322; 7, *Hin*dIII-*Eco*RI digested λDNA. (B) Lanes are as follows: 1, digested H28, showing insert at about 2 kb (arrow); 2, native H28; 3-8, alternately digested and native recombinant plasmids that have no apparent insert; 9, *Hin*dIII-*Eco*RI digested λDNA (bands are: 21.8; 5.24; 5.05; 4.21; 3.41; 1.98; 1.90; 1.71; 1.32; 0.93; 0.84; 0.58 kb from the top.

Figure 7. Recombinant colonies were grown on filters as described, and treated to lyse the cells and denature and fix the DNA to the filter (section 4.2.12). Duplicate filters of each plate were then incubated with either red or green labelled cDNA, the excess label washed off and the filter exposed to Kodak X-ray film. Plates were labelled A to H, with R indicating a red probe and G indicating a green. By comparing the red and green hybridization intensities of each colony, it is possible to pick out colonies that hybridize to the red cDNA only, and so possibly contain ripe fruit specific cDNAs. Each colony was identified according to its grid position as outlined in Figure 5. In all 18 colonies were found to hybridize only, or strongly, to the red.

Figure 8. Minipreparations of clones hybridizing to the red cDNA.

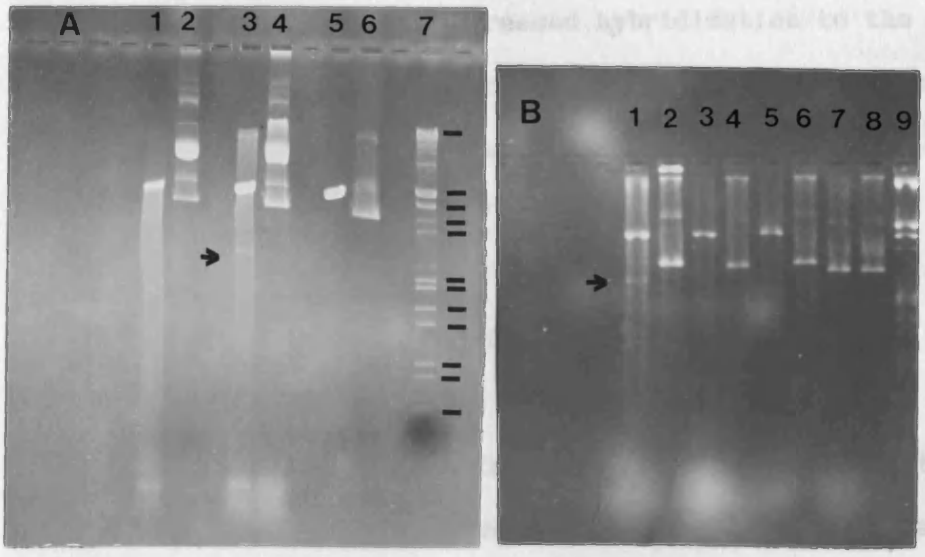


Figure 8. Minipreparations of all red hybridizing clones were made as described in section 4.2.13, digested with *Pst*1 and run on 1.1% agarose gels, alongside a *Hin*dIII-*Eco*RI digested λDNA marker, and both native and linearised pBR322. (A) Lanes are as follows: 1, digested H28; 2, native H28; 3, digested A24, showing an insert at about 2 kb (arrow); 4, native A24; 5, linearised pBR322; 6, native pBR322; 7, *Hin*dIII-*Eco*RI digested λDNA. (B) Lanes are as follows: 1, digested H28, showing insert at about 2 kb (arrow); 2, native H28; 3-8, alternately digested and native recombinant plasmids that have no apparent insert; 9, *Hin*dIII-*Eco*RI digested λDNA (bands are: 21.8; 5.24; 5.05; 4.21; 3.41; 1.98; 1.90; 1.71; 1.32; 0.93; 0.84; 0.58 kb from the top.

fixed to the filter. Double stranded cDNAs derived from red and green fruit were labelled by nick translation as described in section 4.2.12 (ii) (Maniatis *et al.*, 1982). A calculation of probe specific activity is given in Figure 6. cDNA probes were not size selected on agarose prior to labelling as agarose inhibits the nick translation reaction (Maniatis *et al.*, 1982). Pre-hybridization to block non-specific sites was performed as described in section 4.2.12 (iii). The results of the colony hybridization are given in Figure 7. From these results it was possible to select several clones which show increased hybridization to the red fruit derived probe. All clones showing such ripening relatedness were further analysed in terms of size of insert.

4.3.06 Minipreparations of 'ripening' clones.

The method of minipreparation used is shown in section 4.2.13 and was originally obtained from Dr C.M.Lazarus of Bristol University. Only two clones showed detectable inserts, A24 and H28, shown in Figure 8. Discussion of why so few inserts were detected is included in section 4.3.09. The inserts obtained both consisted of two fragments released from the plasmid upon *Pst*1 digestion, a larger fragment of approximately 2 kb, and a smaller digestion product of approximately 200 bp. The similar appearance of

the digested inserts on the gel implies they may be similar in sequence.

4.3.07 Maxipreparations of A24 and H28.

In order to gain sufficient cDNA for further experimentation the clones of interest were subjected to a maxipreparation procedure, designed to greatly increase the amount of insert DNA. Clones of interest were grown up overnight in 10 ml of tetracycline L-broth, and 1 ml of this overnight culture used to inoculate 1 litre of L-broth with 12.5 µg/ml tetracycline. The method eventually used is described in section 4.2.14. Difficulty was encountered with one step of this method, namely lysis of the cells. Initially problems with lysis of the cells were addressed by altering the enzyme batch, which had no positive effect on the problem. Analysis of published methods revealed a variation in lysozyme concentration of 0.65 to 5.00 mg/ml, and a variation of detergent concentration of 0.66 to 2.50% (SDS). I decided to treat my cells with the highest recorded levels of enzyme and detergent, and there was no lysis. These cells were then boiled, and there was still no lysis. A personal communication from P.Valente of Bath University led to strict monitoring of the time the culture was allowed to grow prior to lysis. Apparently the older the cells are the thicker their walls are, and the more difficult they are to lyse. Different methods allow the cells to grow for

different periods of time. The method recommended by Hatfield Polytechnic (Nucleic Acids Workshop) suggests allowing the cells to grow to stationary phase. Dr W.Bains of Bath University recommended harvesting the cells at an OD_{600} of between 0.6 and 0.8. Maniatis *et al.* (1982) gives an OD_{600} of 0.4 for all methods given (boiling, alkali and SDS). The method finally adopted is given in section 4.2.14, and is that recommended by Dr W.Bains (Bath University), except that cell cultures were only allowed to grow to an OD_{600} of 0.4. This was found to be effective, although the yield of insert DNA was low. *Pst*I digested plasmids obtained with this technique are shown in Figure 9. A factor which may have affected cell lysis is the type of growth medium, and this may have been a worthwhile alteration to make.

4.3.08 Northern blotting of end-labelled insert DNA.

The small (approximately 200 bp) portion of each insert was collected and purified as previously described and end-labelled for use in a preliminary Northern blot experiment. The aims of the experiment were to establish the ripening related nature of the inserts, and to practise the method, which was taken from Thomas (1980). End-labelling the 3' overhangs was achieved using T4 polynucleotide kinase, which transfers γ-^{32}P[dCTP] to the protruding single stranded termini.

Figure 9. Maxipreparations of the clones of interest.

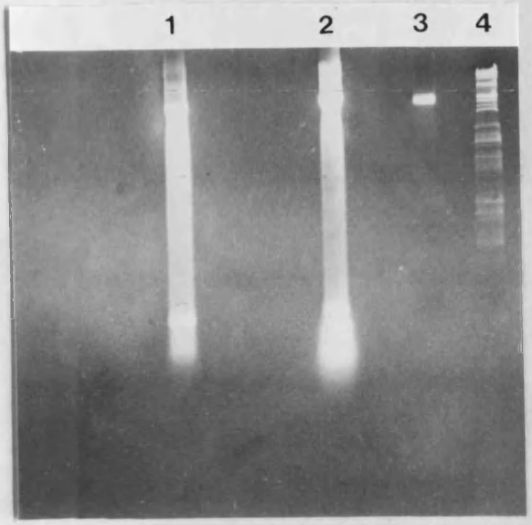


Figure 9. Clones of interest were grown up overnight as described for the miniprep procedure and 1 ml of these cultures used to inoculate 1 l of broth. The maxiprep procedure was carried out as described in section 4.2.14, involving growth in culture and CsCl/ethidium bromide centrifugation to purify the recombinant plasmids. An aliquot of the purified plasmid was digested with *Pst*1, and the digested plasmid run on a 1.1% agarose minigel as described for minipreparations. Inserts were extracted by running onto NA45 paper as described for minipreps. Lanes are as follows; 1, *Pst*1 digested H28; 2, *Pst*1 digested A24; 3, *Pst*1 digested pBR322; 4, *Hin*dIII-*Eco*RI digested λDNA (bands as for Figure 8).

Figure 10. Calculation of the specific activity of end-labelled insert DNA.

I = Incorporated = 571 c.p.m.
T = Total = 120960 c.p.m.

$\frac{I}{B} = \frac{571}{120960} = 0.005 = 0.5\%$ Incorporation

Specific Activity = 571 c.p.m. x $\frac{50\ \mu l}{2}$

= 14275 c.p.m. (0.16 µg)

= 8.9×10^4 c.p.m./µg

Figure 11. Northern blotting of red and green fruit A+ RNA to end labelled cDNA probes.

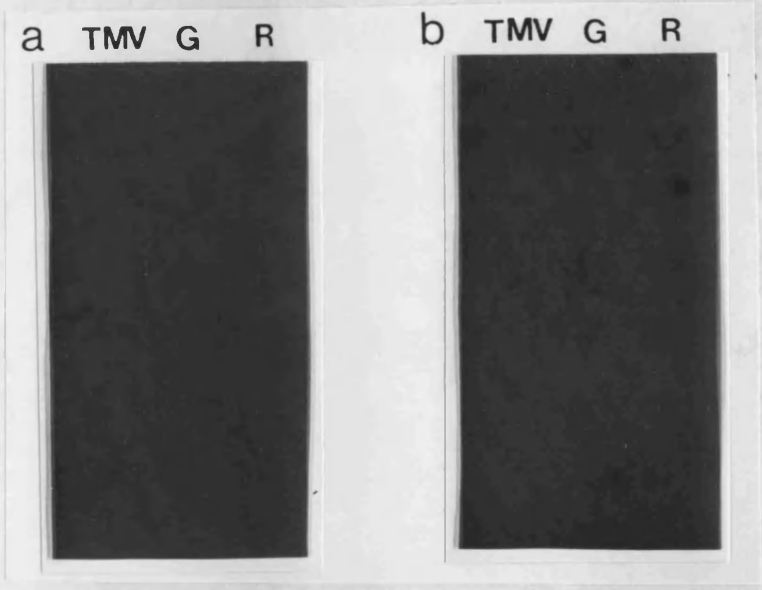


Figure 11. Total fruit RNA was extracted as described in section 3.2.06, selected by oligo (dT) cellulose chromatography, run on a 1.1% sterile agarose gel and transferred to nitrocellulose as described in section 4.2.16. The filter was treated and pre-hybridized as described in that section, and hybridized to the labelled probe. The filters were then washed to remove excess probe, dried, and exposed under X-ray film for 2 weeks. The autoradiographs showed no hybridization, probably due to the low activity of the end labelled probes. Part a was hybridized to the small insert of H28 and part b to the small insert of A24.

G_____CTGCA 3'

3' ACGTC_____G

Labelled probes were used in the hybridization reaction without prior separation of γ-^{32}P[dCTP] residues by passage through Sephadex G50. If hybridization had occurred, the presence of these free nucleotides may have obscured the pattern of hybridization by producing a high background signal. Interpretation would have been compromised by the presence of these nucleosides. Transfer of RNA to the nitrocellulose filters could have been monitored by staining the RNA with ethidium bromide, although this has been shown to reduce efficiency of transfer and would later have had to be omitted (Thomas, 1980). This preliminary Northern blot has been included to show the direction that the work was taking, but represents a very preliminary stage in establishment of the technique. The specific activity of the end-labelled probes is shown in Figure 10, and is low ($9 \times 10_4$ c.p.m./μg). An autoradiograph of each hybridized filter is shown in Figure 11. The experiment was unsuccessful, probably due to poor transfer of RNA from the gel and low specific activity of the 200 bp probes.

4.3.09 'Loss' of clones.

Further work with the ripening related cDNAs was truncated due to loss of inserts from the plasmids. Minipreparations of plasmids from stored bacteria (stored as described in Appendix C, section C.2.04) showed a dramatic drop and then a total absence of detectable insert DNA. A possible cause of insert loss, or lack of inserted cDNA initially, involves the *RecA-* nature of *E.coli* strain HB101. The *RecA+* gene of *E.coli* codes for a product which promotes pairing of single stranded DNA with homologous duplex DNA (Old and Primrose, 1985). Gene expression is induced by the presence of DNA damaging substances, such as nalidixic acid, which suggests that the gene is involved in DNA repair. Peacock *et al.* (1981) showed that homopolymer tailed hybrid plasmids yielded 10-fold fewer recombinants when transformed into a *RecA-* strain, as compared with transformation in a *RecA+* strain, and the *RecA* system is therefore implicated as important for the repair and maintenance of open circular hybrid molecules in *E.coli* cells (Maniatis *et al.*, 1982). This strongly suggests that the incorrect strain was chosen as recipient for the homopolymer tailed hybrid plasmids, which would have had nicks in the DNA duplex, e.g. at the *Pst*I site. A more suitable strain would have had a RecA+ genotype, such as *E.coli* strain RR1.

It is likely that cDNA was correctly synthesized and size selected, as demonstrated by the measurement of incorporation and the appearance of the cDNA on an agarose gel, respectively. Tailing of the cDNA was also demonstrable using the precipitable counts. Suspicion is therefore directed at the annealing procedure. Controls were operated during annealing reactions of dG-tailed plasmid only, and routinely gave zero recombinants, so it is unlikely that the plasmid was re-circularizing with itself. The antibiotic sensitivity of the HB101 cells was not in question as they were tested regularly. The possibility is that no, or very few, cDNA molecules were annealed into the plasmids. Tailed cDNA was annealed to dG-tailed plasmids in the presence of unreacted dCTP nucleosides, and no attempt was made to separate the tailed cDNA. It is therefore possible that unreacted nucleosides hydrogen-bonded to the dG tails and the linear, blunt-ended plasmids then re-circularized. The hybrid plasmids so produced would have a short interruption in the ampicillin resistance gene consisting of a short G/C stretch of 15 to 20 nucleotides. Such plasmids would be selected as apparent recombinants. The two 'ripening related' clones identified as having inserts (A24 and H28) could have contained genuine inserts which were then lost, perhaps as a result of the *RecA*- cell genotype, or the inserts seen initially on gels of minipreparations of the clones, were artifactual.

A further criticism must be applied to the technique of clone selection. Double stranded cDNA clones were nick translated in the presence of ^{32}P[dCTP], and the labelled probes separated from the unreacted nucleosides by ethanol precipitation. The only labelled DNA entering the hybridization reaction was therefore labelled cDNA. If no cDNA inserts were present, this experiment should have revealed it. Instead of this the results appeared to show the presence of clones hybridizing to the red or green fruit derived probes. My suspicions should have been aroused when some clones appeared to hybridize more strongly to the green derived probe (bearing in mind that the library was derived from ripe fruit), but it was believed that these clones represented messages expressed at a low level (but still present) in the red fruit, and at a higher level in the green fruit. It is probable that the hybridizations shown in Figure 7 represent varying levels of background hybridization. The autoradiographic response of colony hybridization appears to depend on the amount of DNA bound to the filter (Grunstein and Wallis, 1979), and therefore the variation seen could be a function of varying levels of cellular DNA. A negative control of an untransformed HB101 colony should have been included on each filter so that the level of background hybridization could be monitored. A better method of screening the library may have been to prepare minipreparations of each test colony, and perform dot blotting experiments rather than colony hybridization.

In this way it would have been possible to standardize the amount of DNA fixed to the filters. The hybridization experiments were useful in that the technique was successfully performed, but the results obtained were probably not interpretable in terms of ripening specific sequences. In spite of the ultimate failure of the experiments, the system for establishing a cDNA library was largely set up in the laboratory. If the experiments were to be repeated the following improvements are suggested: use of a *RecA+* strain of *E.coli* as the host cell; differential colony hybridization to select ripening clones, but with a suitable control colony to allow assessment of background hybridization, or dot blotting to colony derived minipreps as a method of screening; replica plating of colonies using a specially designed tool for replica plating them all at once, as opposed to individual transfer of colonies using sterile toothpicks, which is very time consuming and loads varying amounts of cells; and storage of the library in microtitre plates as opposed to storage of individual clones in eppendorf tubes, for convenience when attempting to access the library.

5.0.00 General Overview.

Fruit ripening has been shown to be a developmental process in avocado and tomato (Christoffersen *et al.*, 1984; Grierson *et al.*, 1985) involving changes in gene expression. During ripening fruit plastids undergo a dramatic transition from chloroplast to chromoplast, with attendant changes in metabolism, pigmentation and ultrastructure, and also alterations in protein complement (Bathgate *et al.*, 1985) and poly A+ RNA complement (Rattanapanone *et al.*, 1978).

The aim of this work was to investigate the changes taking place during ripening in *Capsicum annuum* fruit. Various aspects of change were studied, including a demonstration of change in pigmentation, the changes occurring in chloroplasts and chromoplasts at an ultrastructural level, a comparative study of the changes taking place in the protein populations of unripe and ripe fruit plastids, and a similar study of the polyadenylated RNA complement of green and red fruit, using *in vitro* translation.

The alteration of pigmentation in *C.annuum* fruits during ripening has been studied by Davies *et al.* (1970) and Candela *et al.* (1984). Synthesis of carotenoids is confined to the plastids and elucidation of the carotenoid synthetic pathway, and localization of reactions in *C.annuum* have

largely been performed by Camara and co-workers (Camara, 1984; Camara and Dogbo, 1986; Camara *et al.*, 1982, 1985). The work presented here on pigment change represents a brief study designed to demonstrate and reiterate the established change in pigmentation that occurs during ripening. Photographs showing the visual effects of ripening are presented, and also multiwavelength spectrophotography of extracted Bellboy pigments. This showed the loss of green pigments (chlorophylls) and the accumulation of red pigments (carotenoids) although in the case of Golden Star, the ripe fruit lacks the red pigments, and appears yellow. Change in pigmentation is an obvious ripening associated change, and indicates related change at many levels.

The plastid is the site of dramatic change in ripening fruit. The ultrastructure of *C.annuum* var. Bellboy plastids during ripening has been performed previously (Arundel, 1984), and a description of the changes that take place can be found in Chapter 2. A comparison between Bellboy and two other varieties of *C.annuum* was performed in this work, for the sake of interest, and to correlate ultrastructural features with pigment, protein, and RNA differences. Changes in plastid ultrastructure were clearly demonstrated, with loss of typical chloroplast internal structure and development of chromoplast internal structure, concomitant with the loss of chlorophyll and the appearance of carotenoids. The electron microscopy was unfortunately not

performed at a sufficiently high magnification to observe the fate of plastid ribosomes during ripening. The work of Carde *et al.* (1988) has demonstrated loss of these structures during ripening. These authors looked at the presence and absence of plastid ribosomes during ripening by electron microscopy, and studied the presence and absence of plastid ribosomal RNAs by gel electrophoresis. Results implied loss of plastid ribosomes and rRNAs in fully differentiated chromoplasts, suggesting a lack of protein synthesis in these organelles. Their conclusions are contradicted by the work of Powell and Pryke (1987), who report that isolated chromoplasts incorporate labelled amino acids into novel proteins. Reconciliation of these two contradictory hypotheses is possible if it is assumed that loss of ability to synthesize protein is a gradual process, reaching completion only in the fully differentiated chromoplast. Fruit studied prior to that stage, although they may appear ripe, may retain some residual 'chloroplast' protein synthesis.

An eventual aim was to correlate differences between Bellboy and Lito or Golden Star fruit at the pigment, ultrastructural and protein levels, with differences in the expression of identified ripening related genes. These ripening related sequences were to be identified using a cDNA library, and it was with this future work in mind that

comparative experiments of Bellboy, Lito and Golden Star were carried out.

The most obvious changes associated with ripening (such as ultrastructural and pigment change) occur in the plastids. To look for concomitant changes in protein complement the protein populations of chloroplasts and chromoplasts were subjected to study by three methods (SDS-PAGE, IEF, and 2-Dimensional gel electrophoresis). Considerable variation in the protein complements of unripe and ripe fruit plastids were revealed, as has also been shown for tomato (Bathgate *et al.*, 1985). It was possible to establish the presence of a group of ripening associated products, using each method. Major ripening related products of m wt 33 and 53 kD appeared consistently in SDS-polyacrylamide gels of chromoplast proteins.

Alterations in the protein populations of plastids implied that similar variation may be taking place at the RNA level. Several lines of work (including antibiotic studies; ultrastructural studies; comparisons of nuclear and total RNA in *in vitro* translation profiles; and mutation studies; as outlined in Section 1.1.07) indicated that chromoplasts are almost certainly not active in protein synthesis, which implied that the 'ripening' RNA messages were emanating from the nucleus. Transport of such nuclear encoded proteins from the cytosol to their destination in the plastid, is

brought about by the presence of transit peptides, or leader sequences, at the amino end of the protein. Receptors on the plastid outer membrane seem to recognise the three dimensional structure of these transit peptides, and, in a process requiring energy, cause the proteins to enter the plastid (Ellis, 1977; Keegstra and Bauerle, 1988). Further information in the transit peptide may direct the polypeptide more precisely within the plastid. Such sequences are very important for the chromoplast, as it appears to be unable to synthesize any of its own proteins. In sympathetic conditions ripe red fruit remain on the plant for months, and presumably the chromoplasts are maintained during this time. Maintenance of the chromoplasts within the fruit cells is carried out via a system of transmembrane transport of cytosolic proteins and metabolic intermediates, summarized in Figure 2 of the General Introduction (redrawn from Ziegler *et al.*, 1983).

A study of polyadenylated nuclear transcripts was carried out, preceded by the development of an RNA extraction method from fruit. The study was performed using *in vitro* translation of poly A+ RNAs, and revealed variation at the mRNA level between unripe and ripe fruit, as has also been shown for the tomato (Rattanapanone *et al.*, 1978) and the avocado (Christoffersen *et al.*, 1982). Several products were identified that may have been larger precursors of chromoplast proteins, possessing transit peptides of between

2 and 16 kD. The 33 kD ripening associated product, identified using SDS-PAGE of plastid proteins, was possibly represented as a 49 kD precursor protein in the *in vitro* product profile (see Chapter 3).

Once variation at both the protein and RNA levels was established, the aim was to study expression of a particular ripening associated product in more detail. Towards this end experiments designed to produce a cDNA library from red fruit mRNA were performed. Similar work has been done in tomato (Mansson *et al.*, 1985) and the avocado (Christoffersen *et al.*, 1984).

Future work involves more detailed study of each investigation reported in this thesis, for example of the studies of pigment, ultrastructure, and also physiology of the ripening fruit. The most interesting work, however, involves the investigation of ripening gene expression, possible approaches of which are outlined in the Introduction to Chapter 4. For the reasons given at the end of that Chapter, the work attempted was unsuccessful. Identification of a ripening clone and the subsequent investigations of spatial and temporal gene expression; gene regulation (possibly in relation to ethylene); and function of the gene product(s) remain to be done as future work.

To summarize: the work presented in this thesis demonstrates many changes in the ripening fruit of *C.annuum*. Several products and possibly their precursors have been identified at the protein and mRNA levels. Techniques for the creation of a cDNA library, and the screening of that library for ripening related clones were developed, laying the foundations for future work on expression of specific ripening related genes. This work corroborates previous reports (Grierson *et al.*, 1981; Grierson, 1986) that ripening is a dramatic, developmental process, involving extensive alterations in gene expression.

Appendix A. Use of SP6 RNA polymerase to size RNA bands.

A.1.00 Introduction.

SP6 RNA polymerase is isolated from bacteriophage SP6 infected *Salmonella typhimurium* LT2Z. The enzyme is stable and easy to isolate. It transcribes rightwards from a specific SP6 promoter, which can be linked to a polylinker sequence in a vector, and hence be used to transcribe into RNA the DNA sequence inserted into the polylinker (Melton et al. 1984). RNA produced in this way can be used to increase the sensitivity of nucleic acid hybridization techniques, and to produce RNA substrates for studies on RNA processing and translation. New England Biolabs supply an SP6 RNA polymerase complete with control template DNA to check that the enzyme is transcribing correctly. The template DNA is a pSP64 vector containing the 1.38 Kb *Eco*RI-*Hin*dIII fragment of λDNA, spanning nucleotides 26104-27479, which has been cloned into the polylinker site downstream of the SP6 promoter. This control template DNA was digested in three separate reactions with either *Eco*RI, *Pst*I, or *Sca*I. The resulting three linearized molecules were pooled. Transcription by SP6 polymerase using this control template DNA results in three runoff RNA fragments of discrete size and known sequence. There is a 1.38 Kb fragment corresponding to the *Eco*RI digested DNA, a 0.55 Kb fragment corresponding to the *Pst*I

digested DNA, and a 0.22 Kb fragment from the *Sca*I digested DNA. These RNA fragments were run on a gel as described in section 3.2.11, and used to calculate the sizes of pepper fruit RNA fragments.

A.2.00 Materials and Methods.

A.2.01 Chemicals and reagents.

SP6 RNA polymerase and SP6 control template DNA were obtained from New England Biolabs. Ribonucleotides, spermidine, DTT, RNasin and BSA were of the highest possible purity from Boehringer Mannheim and Amersham International. All other chemicals used were of the highest possible purity available from Sigma, Aldrich and BDH.

A.2.02 Reaction.

The following reaction was set up in a siliconized sterile eppendorf tube: 4.5µl 5X SP6 Buffer (0.2M Tris-HCl, pH 7.5; 30mM $MgCl_2$; 10mM spermidine; 0.5mg/ml BSA), 2.0µl 0.1M DTT and 4.0µl 2.5mM ribonucleotide mix. These reagents were mixed, centrifuged briefly to the foot of the tube and warmed to hand heat (to prevent precipitation of the template DNA). 10.0µl (2µg) of the template DNA was added, and the mixture was mixed and re-centrifuged briefly as before. 1.0µl (to a final

concentration of 1U/µl) of RNasin and 0.5µl (5U) of SP6 polymerase were added. The reaction was incubated at 40°C for an hour. The nucleic acids were then phenol/chloroform extracted as described for cDNA in section 4.2.03 (iv).

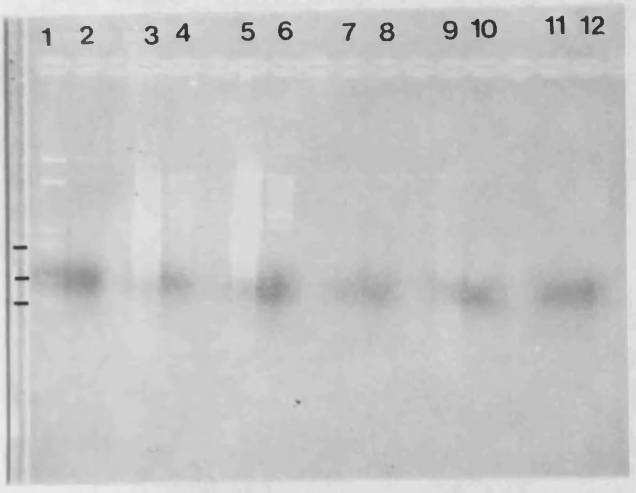
A.2.03 RNA gel.

The SP6 RNA was run against pepper fruit RNA on a 1.1% agarose gel in 10mM phosophate buffer, pH 7.0, as described in section 3.2.11.

A.3.00 Results and Discussion.

Figure 1. Agarose gel electrophoresis of pepper fruit RNA (total and A+) and SP6 marker.

A



B

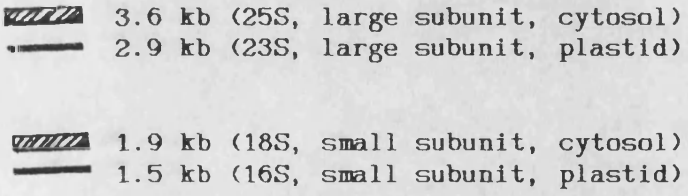


Figure 1. Part a: pepper fruit RNA was run against an SP6 marker RNA as described in section 3.2.11. Lanes are as follows: 1, native SP6 RNA and DNA template; 2, denatured SP6; 3, green fruit total RNA, native; 4, green fruit total RNA, denatured; 5, red fruit total RNA, native; red fruit total RNA, denatured; 6, green fruit A+ RNA, native; 7, green fruit A+ RNA, denatured; 8 and 9, as 6 and 7; 10, red fruit A+ RNA, native; 11, red fruit A+ RNA, denatured. The sizes of the SP6 RNA bands (marked) are 1.38, 0.55 and 0.22 kb from the top. The sizes of the pepper fruit RNA bands were calculated as diagrammed in part b.

Appendix B. RNA extraction methods data.

B.1.00 Introduction.

The results presented in this appendix represent data for the methods of RNA extraction adapted for pepper fruit. Yield and purity became secondary to activity of the RNA in an *in vitro* translation reaction. The detailed methods of the extractions are given in chapter 3, as are references to the sources of the methods. The background to RNA extraction is given in the Introduction to chapter 3. This data has been included to show the data behind choice of the RNA extraction method, on which the results of chapters 3 and 4 depended.

B.2.00 Materials and Methods.

B.2.01 Chemicals and reagents.

All the chemicals and reagents used for the extraction and purification of RNA were of the highest possible purity available from Sigma, Aldrich or BDH. The water was purified through a milliQ system, and also sterilized by autoclaving. All buffers and equipment coming into contact with the RNA were sterilized by autoclaving, dry heat (200°C for 5 hours)

or by soaking in 0.1% DEP in ethanol. Crystalline phenol was redistilled at approximately 160°C, and equilibrated in extraction buffer as described in Maniatis *et al.* (1982).

B.2.02 Extraction methods.

The extraction methods used were (i) GuSCN, as described in section 3.2.03; (ii) phenol extraction and CsCl centrifugation, as described in section 3.2.04; (iii) high SDS method of RNA extraction, as described in section 3.2.05; and (iv) the final method of RNA extraction, as described in section 3.2.06. The yield and purity of the RNA extracted was measured as described in sections 3.2.07 and 3.2.08 respectively. The biological activity of the RNA was tested in a non-optimized *in vitro* translation reaction as described in section 3.2.15. The result of this survey of methods was used to determine the most efficient way to extract total RNA from pepper fruit.

B.3.00 Results and Discussion.

B.3.01 GuSCN Method.

Type of fruit. Mass of tissue. [total RNA]. Abs260/Abs280.

Green	10g	32µg	1.62
Green	10g	76µg	1.80

Green	10g	180µg	1.75
Intermediate	10g	132µg	3.02
Intermediate	10g	72µg	2.30
Red	10g	24µg	1.32
Red	10g	72µg	1.50

average yield = 8µg/g fresh weight SD = 5.00

average purity = 1.90 SD = 0.58

translational activity -

B.3.02 Phenol extraction and CsCl centrifugation.

Type of fruit. Mass of tissue. [total RNA]. Abs260/Abs280.

Green	70g	522µg	1.75
Green	185g	1248µg	1.82
Green	131g	1000µg	1.79
Red	70g	4400µg	1.57
Red	70g	2000µg	1.65
Red	128g	600µg	1.88
Red	87g	670µg	1.60
Red	99g	1420µg	1.59
Red	91g	1720µg	1.68
Red	101g	2376µg	1.30

average yield = 18µg/g fresh weight SD = 17.00

average purity = 1.66 SD = 0.16

translational activity +/-

B.3.03 High SDS method.

Type of fruit.	Mass of tissue.	[total RNA].	Abs260/Abs280.
Green	40g	294µg	2.30
Green	44g	304µg	1.55
Intermediate	20g	117µg	2.00
Intermediate	68g	127µg	4.33
Red	61g	255µg	2.26
Red	76g	529µg	1.80

average yield = 5.6µg/g fresh weight SD = 2.00

average purity = 2.37 SD = 0.99

translational activity -

B.3.04 Final method of RNA extraction.

Type of fruit.	Mass of tissue.	[total RNA].	Abs260/Abs280.
Green	50g	191µg	1.70
Green	50g	319µg	4.00
Green	50g	754µg	2.00
Green	50g	1326µg	1.70
Red	50g	475µg	1.71
Red	50g	396µg	1.82

Red	50g	818µg	1.73
Red	50g	993µg	2.00
Red	50g	627µg	1.93

average yield = 13.11µg/g fresh weight SD = 7.17

average purity = 2.06 SD = 0.73

translational activity ++

Covered more extensively in chapter 3 Discussion, I found the final method of RNA extraction the most useful, in terms of total RNA produced per extraction; yield; purity; and most importantly, translatability.

Appendix C. Optical density and cell density of *E.coli*, storage of cells and preparation of antibiotic stocks.

C.1.00 Introduction.

The two experiments described in this Appendix were carried out as a preliminary to the set of experiments described in Appendix D, which aimed to verify the best method of transformation for the system chosen. The aim of the experiments in this section was to pinpoint the optical density of cell culture which correlated with a particular cell density. The measurements were taken using the cell line of interest, the media and growth conditions to be used in the making of competent cells, and with the spectrophotometer to be used. Cell competence is a transitory phenomenon which occurs between 4 and 9 x 10^7 cells per ml of media for *E.coli* (Hanahan, 1985).

C.2.00 Materials and Methods.

C.2.01 Chemicals and reagents.

Cell line *E.coli* (strain HB101) was obtained from Dr. John Beeching of Bath University. All substances used in the making up of media were obtained from Difco. Other chemicals

were of the highest possible purity available from Sigma, Aldrich and BDH. The antibiotics were from Amersham.

C.2.02 Growth curve of HB101.

10ml of L-broth (1% w/v bacto tryptone; 0.5% w/v bacto yeast extract; 1% w/v NaCl) was inoculated with a scrape from a frozen stock of HB101, and grown at 37°C overnight with moderate shaking. 1ml of this overnight culture was used to inoculate 100ml of L-broth which was then incubated at 37°C with shaking. The time of inoculation was designated time zero, and 1ml samples were taken from the growing culture at regular time intervals, and their Abs 550 measured against an L-broth blank.

C.2.03 Correlation of cell number with OD_{550}.

At each time point described in section C.2.02 10µl of culture was diluted to 10^2 in 1ml of L-broth, and 10µl of this dilution diluted to 10^4 in a further 1ml of L-broth. 10µl of this final dilution was spread onto an L-agar plate (so that the colonies grown represented a 10^6 dilution. The plates were incubated at 37°C inverted overnight.

C.2.04 Storage of *E.coli* strain HB101.

Cells were grown in a 10ml overnight as described in section C.2.02, and 0.8ml of culture was added to 0.2ml of sterile glycerol. The culture and glycerol were mixed and the cells flash frozen in a dry ice and ethanol bath. The frozen stocks were stored at -80°C. Care was taken to ensure that the cells did not thaw out when samples from the stocks were taken as scrapes from the surface. Repeated thawing and re-freezing would have resulted in eventual loss of cell viability.

C.2.05 Preparation of antibiotic stocks.

Antibiotic stocks were prepared and stored as in Maniatis (1982). Ampicillin (sodium salt) was made to 25 mg/ml in pure water and sterilised by filtration. The stock solution was stored in aliquots at -20°C. The working concentration was between 35 and 50 µg/ml.

Chloramphenicol was made to 34 mg/ml in ethanol and stored at -20°C. The working concentration was 170 µg/ml for the amplification of plasmids.

Tetracycline hydrochloride was made to a 12.5 mg/ml in ethanol:water (1:1 v/v), and stored in the dark at -20°C. The working concentration used was 12.5-15 µg/ml.

C.3.00 Results and Discussion.

Figure 1. Growth curve of HB101 in L-broth and correlation of optical density and cell density.

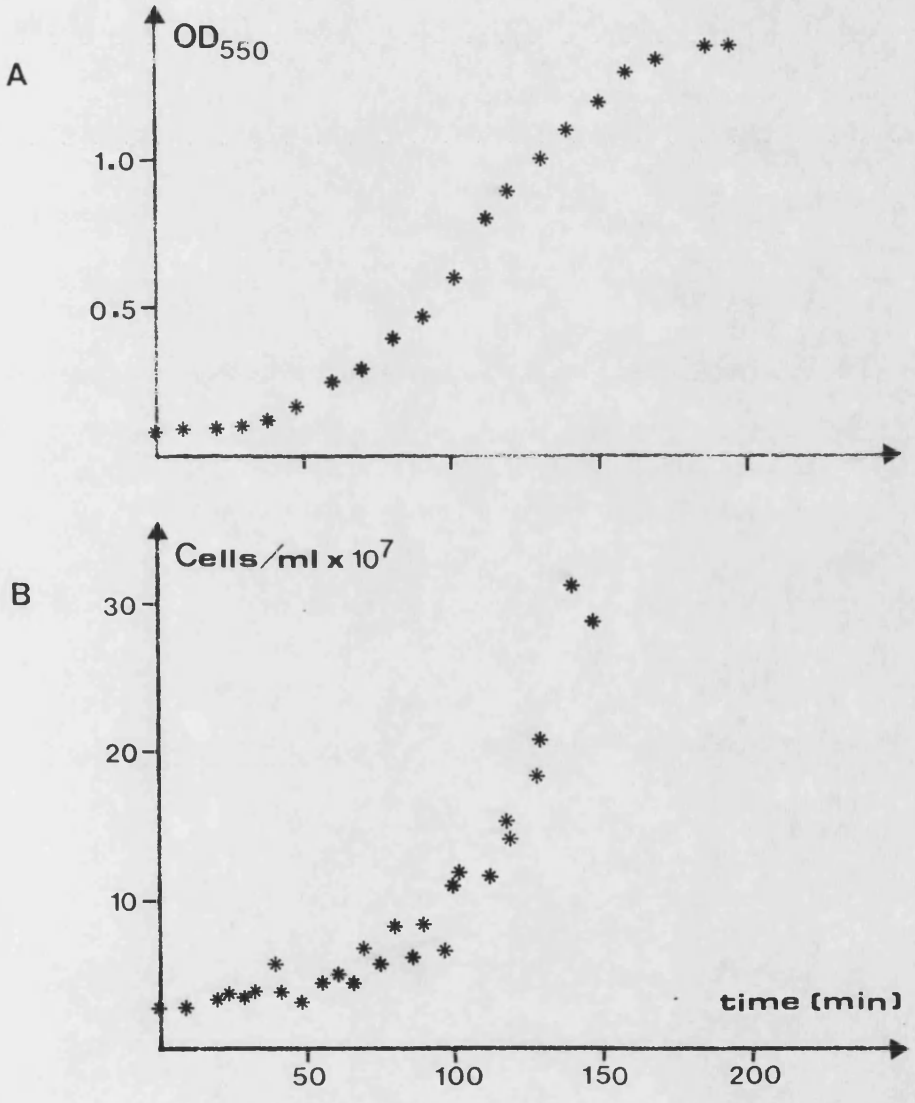


Figure 1. (A) An overnight culture of *E.coli* strain HB101 was used to inoculate a culture, at time = 0. 1 ml samples were taken regularly and the variation of optical density with time was measured. The curves were each obtained with points from two experiments. (B) Variation of cell density with time was measured as described in section C.2.03. Using both curves it is possible to estimate the cell density, from the optical density.

Cell densities of 4-9 x 10^7 cells per ml are obtained at times of 65 to 100 mins after inoculation of a 100 ml culture with 1 ml of a 10 ml overnight culture of *E.coli* strain HB101. This cell density correlates with an optical density at 550 nm of 0.52.

Appendix D. Survey of transformation methods.

D.1.00 Introduction.

$CaCl_2$ mediated uptake of DNA into *E.coli* was first reported by Mandel and Higa (1970), who were interested in the effects of monovalent and divalent cations on cell wall permeability of *E.coli*, and correlation with DNA uptake. They found that DNA uptake into the cells depended on the presence of calcium ions, and was enhanced by chilling at a low temperature. The method they developed is the basis of most successive methods of cell transformation. In 1972 Cohen *et al.* showed that *E.coli* would incorporate circular pieces of DNA, carrying drug resistance genes if treated as recommended by Mandel and Higa (1970). They demonstrated that the transformed cells needed a period of recovery before being exposed to the relevant drug to allow them time to express the resistance gene. A paper by Norgard *et al.* (1978) explored the optimization of parameters required to transform efficiently the highly enfeebled strain of *E.coli*, χ1776. These workers demonstrated the importance of the state of growth of the cells prior to $CaCl_2$ treatment, and the importance of buffer and medium constituents, and cation concentrations. They showed that transformation efficiency was also affected by plating quantities and temperature. Dagert and Ehrlich (1979) found the transformation efficiency much enhanced by an overnight incubation at 4°C in $CaCl_2$ solution,

and it is their method which is recommended by Maniatis *et al.* (1982). Douglas Hanahan (1985) performed a survey of transformation methods, in which the effects of various cations and various combinations of cations were studied. He found that a positive effect was produced by an elevated level of Mg^{2+} in the culture medium, by prolonged incubation in Mn^{2+}, Ca^{2+}, Rb^{+}, or K^{+}, by the presence of DMSO, DTT or $HACoCl_3$ (hexamine cobalt III chloride) and a short heat pulse performed after the addition of the DNA. His work produced several methodologies, each suitable for different cell lines, or tasks (Hanahan, 1985) and having different transformation efficiencies.

In order to determine the most efficient transformation method using the reagents, water, and equipment available, five of Hanahan's methods were surveyed using the *E.coli* strain HB101 and pBR322 which carries resistance to tetracycline and ampicillin, and comprised the host and vector system to be used in the creation of the cDNA library. HB101 is suitable for all the types of transformation used. The initial work involved characterization of the growth of HB101 cultures, which is described in Appendix C. It is important to take the growing cultures at the mid-log phase of growth, and this can be estimated from the OD_{550} if the optical density corresponding to a particular cell density is known, calculatated using a particular spectrophotometer. Initially problems with positive controls were encountered, that is,

untransformed competent cells grew on the tetracycline plates. This could have been caused by light inactivation of the antibiotic in the plates, the presence of a Tet^R population in the HB101 culture, the presence of Mg^{2+} in the medium inactivating the antibiotic, or a faulty antibiotic stock. Experiments showed that the HB101 had no resistance to either tetracycline or ampicillin and a similar result was obtained with fresh plates, implying that antibiotic inactivation in the plates was unlikely. It was decided to leave magnesium out of the medium, even though it was reputed to improve transformation frequencies, and a new tetracycline stock was sought, and aliquoted in small amounts to avoid freezing and thawing. The simple method recommended by Hanahan (1985) initially produced the highest transformation frequencies, but using acid-washed glassware and a highly purified source of water increased all the transformation frequencies and allowed further comparison. It was found to be important to leave the transformed cells in a drug-free environment for the maximum length of time (1 hour) for resistant colonies to develop.

D.2.00 Materials and Methods.

D.2.01 Chemicals and reagents.

E.coli strain HB101 was obtained from Dr John Beeching of Bath University. Plasmid pBR322 was grown in HB101 and harvested in a maxiprep procedure as described in section 4.2.14. The

purity and integrity of the plasmid preparation was checked on a gel, and the concentration of the plasmid estimated from its absorbance at 260nm as described in section 3.2.07 for RNA, except that an Abs_{260} of 1 corresponds to 50µg/ml for DNA. The plasmid was stored in water at -80°C.

All chemicals used in the preparation of buffers used to make competent cells were of the highest possible purity from Aldrich, Boehringer, Sigma or BDH. Glassware was acid-washed prior to use so that contaminants would not interfere with the procedures. The water used was purified through a milliQ system and autoclaved to sterilize. 10% v/v sodium hypochlorite solution was used to sterilize plasticware that could not be autoclaved, and also for the safe disposal and decontamination of equipment.

D.2.02 Simple transformation.

A 10ml overnight culture of HB101 was grown as described in section C.2.02 and 1ml of this culture was used to inoculate 100ml of L-broth. This culture was grown shaking at 37°C until the culture was midway through the logarithmic phase at a cell density of 4-9 x 10^7 viable cells per ml, which took about 110 minutes. The culture was collected in sterile Sorvall 50ml polypropylene tubes and chilled on ice for 10-60 minutes. The cells were pelleted by centrifugation at 4000 r.p.m. in a Sorvall 8 x 50ml head at 4°C for 15 minutes. The supernatant

was decanted and the tubes drained by tapping on paper towels. The cells were dispersed in 1/3 of the original culture volume of SB, simple transformation buffer, (100mM KCl; 50mM $CaCl_2.2H_2O$; 10mM K-MES, pH 6.3; to a final pH of 6.2). The K-MES (2[N-morphino]ethone sulphonic acid) was prepared as a 0.5M MES solution that had been equilibrated to pH 6.3 using concentrated KOH, and sterilized by filtration through a 0.22µl membrane. The cells were left on ice for 10-60 minutes and pelleted by centrifugation as before. The pellet was drained and the cells resuspended in 1/12.5 of the original culture volume of SB. 200µl aliquots were pipetted into Eppendorf tubes, and the DNA added in a volume of less than 20µl. To determine the transformation frequency of competent cells prepared by the method, 1ng of pBR322 DNA was added in 2µl. The tubes were incubated on ice for 10-60 minutes, heat shocked at 42°C for 90 seconds and chilled by returning immediately to ice. 800µl of growth medium was added, and the cells incubated at 37°C with moderate agitation for 30-60 minutes.

D.2.03 Standard (high efficiency) transformation.

100ml of L-broth culture was grown to a cell density of 4-7 x 10^7 viable cells per ml, as described in section D.2.02. The culture was collected in Sorvall 50ml tubes and chilled on ice for 10-15 minutes, before centrifugation at 4000 r.p.m. in a Sorvall 8 x 50ml head at 4°C for 15 minutes. The pellet was

drained thoroughly and resuspended in 1/3 of the original culture volume of TFB, standard transformation buffer, (100mM KCl; 45mM $MnCl.4H_2O$; 10mM $CaCl_2.2H_2O$; 3 mM $HACoCl_3$, pH 6.3; to a final pH of ≈ 6.2). The cells were incubated in TFB on ice for 10-15 minutes, and re-pelleted as before. The pellet was resuspended in 1/12.5 of the original culture volume of TFB. DnD solution (1M DTT; 90% v/v DMSO; 10mM potassium acetate, pH 7.5) was added to 3.5% v/v, that is 7µl per 200µl of cell suspension. The cells were incubated on ice for a further 10-20 minutes, and a second aliquot of DnD solution was added to give a final concentration of 7% v/v. The cells were incubated for another 10-20 minutes, and then 210µl aliquots were pipetted into Eppendorf tubes. DNA solution was added in a volume of less than 20µl as described in section D.2.02, and the cells incubated on ice for 20-40 minutes. A heat shock and chilling step were carried out as in section D.2.02, and the cells grown in 800µl of medium as described in that section.

D.2.04 Frozen storage of competent cells, protocol 1.

(i) Preparation of competent cells.

A 100ml culture was grown as described in section D.2.02 until the cell density reached about 4-9 x 10^7 viable cells per ml. The culture was collected in Sorvall 50ml centrifuge tubes and chilled on ice for 10-60 minutes, prior to centrifugation at 4000rpm in a Sorvall 8 x 50ml head, at 4°C for 15 minutes to

pellet the cells. The pellet was drained and the cells resuspended in 1/3 of the original culture volume of FB, simple frozen storage buffer, (100mM KCl; 50mM $CaCl_2.2H_2O$; 10% w/v redistilled glycerol; 10mM potassium acetate, pH 7.5; to a final pH of 6.2 using 0.1M HCl). Potassium acetate was prepared as a 1M stock, made to pH 7.5 with KOH and sterilized through a 0.22μm membrane. The suspension was incubated on ice for 10-60 minutes and pelleted as before. The pellet was resuspended in 1/12.5 of FB and 200μl aliquots of the suspension were transferred to Eppendorf tubes and flash-frozen in a dry ice/ethanol bath for several minutes. The competent cells were stored at -80°C until required.

(ii) Use of competent cells.

The tubes were removed from the freezer and allowed to thaw at room temperature until the cell suspension became liquid, and were then transferred to ice. The DNA solution was added in a volume of less than 20μl and swirled to mix. The tubes were incubated on ice for 10-60 minutes, and then given a heat shock at 42°C for 90 seconds, and re-chilled on ice. 800μl of growth medium was added and the cells incubated with moderate shaking at 37°C for 30-60 minutes.

D.2.05 Frozen storage of competent cells, protocol 2.

A 100ml culture was grown to a viable cell density of 6-9 x 10^7 cells per ml as described in section D.2.02. The cells were collected in sterile Sorvall 50ml tubes and chilled on ice for 12-15 minutes. The cells were pelleted by centrifugation at 4000 r.p.m. in a Sorvall 8 x 50ml head at 4°C, for 15 minutes. The pellet was thoroughly drained and resuspended in 1/3 of the original culture volume of FSB, frozen storage buffer, (100mM KCl; 45mM $MnCl_2.4H_2O$; 10mM $CaCl_2.2H_2O$; 3mM $HACoCl_3$; 10mM potassium acetate, pH 7.5; 10% w/v redistilled glycerol; to a final pH of ≈ 6.2 adjusted with 0.1M HCl). The cell suspension was chilled on ice for 10-15 minutes, and re-pelleted as before. The pellet was resuspended in 1/12.5 of the original culture volume of FSB. DMSO was added to 3.5% v/v, and the cells chilled for 5 minutes. Another aliquot of DMSO was added to a final concentration of 7% v/v and incubated on ice for a further 10-15 minutes. The suspension was then aliquoted in 210µl volumes into Eppendorf tubes and flash-frozen in a dry ice and ethanol bath for several minutes. The cells were stored at -80°C and used as described in section D.2.04 (ii).

D.2.06 Frozen storage of competent cells, protocol 3.

A 100ml culture was grown to a viable cell density of 4-7 x 10^7 cells per ml as described in section D.2.02. The cells were collected in sterile Sorvall 50ml tubes and chilled on ice for 12-15 minutes. The cells were pelleted by

centrifugation at 4000 r.p.m. in a Sorvall 8 x 50ml head at 4°C, for 15 minutes. The pellet was thoroughly drained and resuspended in 1/3 of the original culture volume of RF1 (100mM KCl; 50mM $MnCl_2.4H_2O$; 30mM potassium acetate, pH 7.5; 10mM $CaCl_2.2H_2O$; 15% w/v redistilled glycerol; to a final pH of 5.8 adjusted with 0.2M acetic acid). The cells were incubated on ice for 1 hour (less time is needed for cell lines other than HB101) and pelleted as before. The drained pellet was resuspended in RF2 (10mM MOPS, pH 6.8; 10mM KCl; 75mM $CaCl_2.2H_2O$; 15% w/v redistilled glycerol; to a final pH of 6.8, adjusted with NaOH). The MOPS (was prepared as a 0.5M stock, pH 6.8. The cells were incubated on ice for 15 minutes and then flash-frozen in a dry ice and ethanol bath for several minutes. They were stored at -80°C. The cells were used as described in section D.2.04.

D.2.07 Calculation of transformation frequency.

Transformation frequency is defined as the number of transformants per µg of DNA added. 200µl aliquots of competent cells from each of the five methods described were transformed with 1ng of pBR322 DNA. Known volumes of the 1ml final solution were plated out onto L-agar plates (1% w/v bacto tryptone; 0.5% w/v bacto yeast extract; 1% w/v NaCl; 1.5% w/v bacto agar) containing either 12.5µg per ml tetracycline or 50µg per ml ampicillin. The plates were incubated inverted at 37°C overnight, and the colonies counted in the morning. The

amount plated was a percentage of the whole, and the total number of potential colonies could be calculated, and expressed in terms of c.f.u. (colony forming units) per µg of DNA added.

D.3.00 Results.

Protocol.	cfu when 10µl plated			Efficiency.
	Expt 1	Expt 2	mean	(c.f.u./µg DNA)
fs 1	59	48	53.5	5.35×10^6
fs 2	36	33	34.5	3.45×10^6
fs 3	111	119	115.0	1.15×10^7
Simple	51		51	5.10×10^6
	cfu when 100µl plated			
	Expt 1	Expt 2	mean	
DnD	355	299	327	3.27×10^6

D.4.00 Discussion.

Competence is a transitory phenomenon, which reaches a peak in *E.coli* at the mid to late log phase of growth. No difference is reported by growing the cells in different rich media, and DNA sequence also appears to have no effect on transformation efficiency (Hanahan, 1983).

The addition of Mg^{2+} to the growth medium has been shown to increase the transformation efficiency 15-20 fold, and other divalent cations also have a positive effect. DMSO has a stimulatory effect in the presence of divalent cations; DTT and $HACoCl_3$ have positive effects independently of the presence of other substances. Hanahan (1983) proposes that transformation involves two steps, uptake of DNA and establishment of the DNA in the cell, with establishment being the limiting step. The kinetics of transformation are incompatible with a model of passive diffusion through pores in the cell surface, and specific channels are suggested. Divalent cations and DMSO may aid transformation by shielding phosphates and solvating the metallic cations respectively, so bringing together the otherwise unlikely association of two phosphate rich compounds, and stabilizing and organizing ionic interactions on the boundary between the lipid bi-layer and the aqueous environment.

At low temperatures the fluidity of membranes becomes reduced. Temperatures of around 0°C and the presence of divalent cations are necessary to produce phase changes in synthetic membrane vesicles (Verkleij *et al.*, 1974). Hanahan (1983) proposes that divalent cations, DMSO and low temperatures act together to crystallize parts of the bacterial envelope to allow the entry of DNA, possibly through conformationally altered pores or channels. Divalent cations, particularly Mg^{2+}, may cause the re-orientation of lipopolysaccharide molecules away from such pores thus allowing the entry of DNA molecules. Hanahan suggests that $HACoCl_3$ acts as an analog of vitamin B12, for which specific channels exist in the cell envelope. The $HACoCl_3$ is possibly binding to the pores, altering them in such a way as to allow the entry of DNA. Vitamin B12 itself has been shown to be a potent inhibitor of transformation, lending credence to this suggestion.

The method of transformation found to be the most successful was Hanahan's Frozen Storage, protocol 3, not the standard high efficiency transformation as expected. FS 3 routinely gave efficiencies in the order of 10^7, whereas the standard procedure gave efficiencies an order of magnitude lower, 10^6. A possible explanation for this lies in the extreme sensitivity of the standard method to impurities in the water, the DMSO or contamination of the glassware. Contamination of the water is possible even though it was filtered through a Millipore MilliQ reverse osmosis system, as the cartridges

gradually become saturated, and the contamination with organic substances increases although the resistivity of the water remains high. DMSO breaks down into dimethyl sulphone and dimethyl sulphide, the latter being a potent inhibitor of transormation. The DMSO was treated for over an hour prior to use with gaseous nitrogen in an attempt to remove some of the more volatile oxidation products, but perhaps this was insufficient. Other workers re-distill their DMSO (John Beeching, personal communication) and this probably should have been attempted to see if it improved transformation frequencies.

Overall low frequencies were probably due to the omission of Mg^{2+} from the culture media. This was done in an attempt to prevent tetracycline inactivation, although it is unlikely that Mg^{2+} in the media could have had such a far-reaching effect. In retrospect Mg^{2+} should have continued to be included in the culture media, and it was probably a mistake to omit it. The transformation frequencies of stored cells were observed to drop with time in storage and so cells used for the establishment of the cDNA library were usually prepared only a few days before they were required.

The method of transformation used as a result of this work was the Frozen Storage Method, protocol 3.

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